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Toxicity of Chemical Constituents of PM10 in the South Coast Air Basin of California

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY



AIR RESOURCES BOARD
RESEARCH DIVISION

**TOXICITY OF CHEMICAL CONSTITUENTS
OF PM₁₀ IN THE SOUTH COAST
AIR BASIN OF CALIFORNIA**

Final Report
Contract No. 93-318

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Abstract

The purpose of this study was to evaluate responses to inhalation of particle-gas mixtures (PM) that have been implicated as potentially causing human mortality and morbidity using aged (22 to 24 months) rats. Controlled (4 hr/day, 3 d/wk, 4 wk) nose-only exposures were performed. PM is a complex mixture of particles with different sizes and chemical compositions. Two components of the fine ($\leq 2.5 \mu\text{m}$) particle fraction of PM in California, carbon (C; $50 \mu\text{g}/\text{m}^3$) and ammonium bisulfate (ABS; $70 \mu\text{g}/\text{m}^3$) were tested alone and in mixtures with ozone (O_3). The particles were about $0.3 \mu\text{m}$ mass median aerodynamic diameter. Control atmospheres were purified air and O_3 alone. This study was performed in two phases. Phase 1 examined the effects of selected PM components, alone and in mixtures, on mechanism-related endpoints. Phase 2 examined the effects of a mixture of ozone and particles as a function of particle concentration. Exposures to particle-containing atmospheres produced lung injury, measured as the labeling of DNA in lung epithelial and interstitial cells undergoing replication at the end of the 4-week exposure period. However, group mean value differences were significant only in the group of rats exposed to the atmosphere containing a combination of ABS, O_3 and C, compared to the purified air controls. Ozone increased cell replication rates 20 to 40% in interstitial and epithelial cells, but cell replication rates in rats exposed to particle plus ozone mixtures were increased by between 250 to 340%, relative to purified air. Macrophages from rats exposed to PM components exhibited slightly, but not significantly, depressed ability to attack antigenic material (SRBC) via Fc receptor-mediated processes when measured immediately post-exposure but were reduced significantly and in a dose-dependent manner when measured 24 hr post-exposure. Macrophage production of a potentially toxic free radical (superoxide) was immediately increased by exposures to the ABS + C + O_3 . The effects of this mixture were significantly greater than those observed for O_3 alone. However, 24-hr post-exposure superoxide production was depressed compared to controls ($p \leq 0.1$), suggesting that the time-course of response should be examined further. None of the exposures significantly increased permeability (measured as bronchoalveolar lavage (BAL) fluid concentrations of either total protein or albumin), immediately post-exposure, however permeability measured 24 hr after exposure was increased ($p \leq 0.05$). These results demonstrate that PM exposures can adversely affect biological responses that are relevant to mechanisms that have been postulated to explain the epidemiological associations between ambient PM exposures and increased human morbidity and mortality. The effects of PM were amplified when ozone, an oxidant gas, was present. This suggests that other oxidants could also interact with PM and future studies should evaluate this possibility. Studies of mechanisms of action of PM and PM mixtures should be performed to identify the processes leading to injury at the molecular level. This can be done using transgenic and other sensitive animal models. There remain uncertainties with respect to identifying components of PM with respect to composition and size that may cause human health effects. Future studies are needed to address these uncertainties.

Executive Summary

Background

The purpose of the study was to evaluate toxicologically relevant responses to particle-ozone mixtures found in California's air that have been implicated as potentially causing toxic effects in humans. Although epidemiological evidence has associated ambient exposures to particulate air pollution (PM) with increases in human acute illnesses and deaths, there remain important uncertainties or concerns. Among these uncertainties are questions regarding the identity of the causal agents with respect to particle size or composition, the paucity of data on ambient fine particle concentrations (particles smaller than 2.5 μm in aerodynamic diameter), and the relative influences of other pollutants and meteorological variables that might confound the epidemiological associations. There are also uncertainties as to the mechanisms by which PM might induce toxic effects. Some of the pulmonary-related mechanisms that have been postulated include increased lung permeability or "leakiness" leading to edema, production of excess or thickened mucus, alterations of anti-infection host defenses, and lung inflammatory responses with changes in lung structure or cells. Data relevant to California that address PM-related uncertainties and mechanisms in a controlled and rigorous manner can support the Air Resources Board's establishment of ambient air quality standards to protect human health. This report presents the results of a controlled laboratory inhalation study that was conducted with the support of the Air Resources Board to evaluate the effects of particle inhalation on biological responses relevant to those mechanisms.

Methods

This state-of-the-art inhalation study examined biological endpoints that could be associated with certain of the possible mechanisms for PM-induced toxicity using aged rats. Aged rats were used as laboratory models of older people in the human population. People older than 65 years of age, or people with pre-existing lung or heart disease have been reported to be especially susceptible to PM-induced toxic effects. The PM components that were studied were elemental carbon (C) and ammonium bisulfate (ABS), both of which are important constituents of submicron aerosols in California. The concentrations used were low, compared to those commonly used in toxicology studies, and were based on estimates of peak 4 hr levels for these components in California ambient air. These levels were 50 $\mu\text{g}/\text{m}^3$ for C and 70 $\mu\text{g}/\text{m}^3$ for ABS for low-level exposure and twice these values for high-level exposures. Some of the atmospheres tested included 0.2 ppm ozone (O_3). The biological responses studied were related to the pathogenesis of lung diseases. Some of the responses studied, such as cell replication, are part of normal injury-repair processes, while others, such as cellular immunological measures, are more related to defense mechanisms.

The project was performed in two Phases. Phase 1 examined the effects of specific PM components, alone and in mixtures, on mechanism-related endpoints. The primary hypotheses tested were: (1) that acid coated insoluble particles ($\leq 1 \mu\text{m}$ MMAD) would provoke deleterious responses in aged rats; and (2) that ozone (a pollutant frequently present in urban atmospheres) would potentiate the toxicity of these particles. Inhalation studies were therefore performed to determine the relative toxicities of individual components and mixtures of these PM components, in the presence and absence of O_3 . In Phase 2 of the study, the role of particle concentration on biological responses and mechanisms were then examined using the PM mixture from Phase 1 that induced the most significant effects. The goal of the second Phase was to determine whether the effects of inhaled particle mixtures on mechanism-related endpoints varied as a function of particle

dose. The hypothesis tested in Phase 2 was (3) lung injury or responses induced by PM-related mechanisms would intensify as a function of particle concentration.

Results

The most significant finding of the Phase 1 experiments was that although exposures to particles alone caused some changes in the tested endpoints, only those atmospheres that contained a mixture of particles plus O₃ produced statistically significant responses. This is especially important for air pollution issues in California where high concentrations of particles and O₃ are likely to be co-present in urban atmospheres. The Phase 2 studies examined the particle plus O₃ interaction as a function of particle concentration. An unexpected finding was that for nearly all of the biological endpoints examined, the responses that occurred in animals exposed to a lower dose of particles were not significantly different from those in rats exposed to a dose of particles that was twice as high. This lack of significant increase in response with increased dose could represent a saturation of response mechanisms, or it may reflect dose-dependent differences in the mechanisms of action, differences in the time-course of responses, or to an inhibition of normal defense reactions at higher exposures, as the endpoints used are correlated with defenses that serve to protect the organism.

The specific results in the Phase 2 study varied according to endpoint. When lung cells are injured, adjoining cells can divide to replace them. The location and intensity of PM-induced lung injury was measured in this study using a chemical to label the DNA of the dividing lung cells and comparing the numbers of dividing cells in the lungs of PM plus O₃-exposed and control rats. The numbers of labeled cells in PM plus O₃-exposed rats were increased at both particle dose levels compared to those in lungs from either purified air or O₃-exposed rats. Ozone modestly (20 to 40%) increased cell replication, but cell replication in the lung of PM plus O₃-exposed rats was increased 250 to 340%, relative to purified air. Increased permeability may be associated with increased lung reactivity or inflammatory responses, however in this study none of the exposures resulted in increased infiltration of inflammation-related cells into the lung. The lungs of rats exposed to the PM plus O₃ mixtures in Phase 2 were more permeable to proteins than those of controls or single pollutant-exposed rats. Macrophages are an important component of cell-mediated host defenses against infections. When macrophages are injured, their ability to recognize and destroy pathogens is diminished, and toxic compounds generated by injured macrophages can be released into the lung and thus injure other cells. In this study, macrophage's ability to identify and bind antigens was reduced in a dose-dependent manner for particle plus O₃ mixtures. The production and release of free radicals by macrophages from PM-exposed rats was elevated immediately after exposures, but was below control levels 24 hr after exposure. This study also demonstrated small increases in mucus production in PM-exposed rats, however the changes were small and not statistically significant.

Conclusions

In this study aged rats were exposed by inhalation to relatively low concentrations of particles that are major components of ambient air pollution in California. Biological responses were evaluated that could be linked to postulated mechanisms by which inhaled particulate matter might induce toxicity in humans. PM-exposures under controlled laboratory conditions caused statistically significant, and perhaps biologically significant, injury to lung cells and caused functional depression of an important component of the lung's immune system. It is important to remember that the animals used in this study were old for their species, but were otherwise in

good health (mortality in controls was 5/100 over 1 month). Despite this, statistically significant changes in biological responses that can be related to plausible mechanisms for PM-induced toxicity were demonstrated, but almost exclusively in atmospheres that contained both PM and O₃. Although O₃ is an important oxidant, other ambient oxidants such as nitrogen oxides, peroxides and transition metal should be evaluated in future studies. The particles evaluated in this study were about 0.3 µm in diameter. Studies of the dependence of particle toxicity on both size and concentration are needed to identify components of ambient PM that may be causally linked to health effects. Future studies should also include the use of transgenic or other animal models that simulate the characteristics of human lung and heart diseases, evaluations of the chronic effects of PM exposures and studies of the time-course of PM-induced injuries.

Introduction

Human exposures to particulate matter (PM) have been associated with acute illness and death, especially in people older than 65 years of age or people with pre-existing lung or heart disease. Current federal and California ambient air quality standards were established for particles smaller than 10 μm (PM10) in mass median aerodynamic diameter (MMAD), because particles in this size range are inhaled and penetrate deeply into the respiratory tract, and they are associated with adverse effects on human health. The California and federal PM10 standards were promulgated to (a) prevent excess deaths from short-term exposures and from exacerbation of symptoms in sensitive individuals with respiratory disease; and (b) prevent excess seasonal declines in pulmonary function, especially in children. The current National Ambient Air Quality Standard (NAAQS) for PM10 is 50 mg/m^3 (annual arithmetic mean) and 150 $\mu\text{g}/\text{m}^3$ (24 hr arithmetic mean). The State of California has set more stringent standards of 30 $\mu\text{g}/\text{m}^3$ (annual geometric mean) and 50 $\mu\text{g}/\text{m}^3$ (24 hr arithmetic mean concentration). Recent epidemiological studies have raised concerns that the current Federal standards are not sufficiently protective and the U.S.E.P.A. have recently proposed standards to specifically limit concentrations of ambient particles less than 2.5 μm MMAD (PM2.5). PM2.5 concentrations are strongly influenced by emissions from combustion sources and atmospheric chemical reactions, whereas larger particles ($>2.5 \mu\text{m}$) are primarily generated by mechanical processes such as abrasion and resuspension.^{1,2} The chemical constituents of the larger sized particles are predominantly those characteristic of the earth's crust (e.g. iron, silica, aluminum). The PM2.5 particles are more enriched in heavy metals, elemental and organic carbon, and products of atmospheric chemical and photochemical processes including sulfate and nitrate salts.^{3,4}

Exposures to urban PM10 at levels near the State's (50 $\mu\text{g}/\text{m}^3$, 24 hr average) and often below the national (150 $\mu\text{g}/\text{m}^3$, 24 hr average) ambient air quality standard (NAAQS) are associated with increased hospital admissions and emergency room visits for respiratory illnesses,^{5,6,7,8} increased incidences of asthma attacks,^{9,10} increased asthma medication use,¹¹ reduced pulmonary function,^{11, 12,13} and increased daily mortality.^{14,15,16,17} People whose deaths have been attributed to PM10 exposures have usually been older than 65 years of age, although health effects have also been reported for children and people with asthma. Individuals with pre-existing cardiovascular or chronic pulmonary diseases are among those with elevated risk of adverse PM-related effects.¹⁸

Other pollutants, such as ozone (O₃), sulfur dioxide (SO₂), nitrogen dioxide (NO₂) and carbon monoxide (CO) have been examined in conjunction with PM health effects in some studies, as have meteorological variables such as temperature, relative humidity and season. Interactions of O₃ or CO with PM-induced health effects have been reported and there is some contention that in some studies it is not possible to disentangle the effects of the co-pollutants from those of PM.

PM₁₀ standards are mass-based and do not consider particle composition with respect to the health effects observed. Nor do they differentiate between the effects of fine particles with diameters (d_p) < 2.5 μ m and coarse particles ($2.5 \leq d_p \leq 10 \mu$ m). Some data suggest that associations between mortality and fine particle or acidic particle concentrations are stronger than those between mortality and coarse particle concentrations.¹⁹ For example, exposures to respirable fine ($\leq 2.5 \mu$ m MMAD) particles and sulfates have been associated with increased total annual mortality rates.^{20,21} However, other data suggest a role for the coarse particle fraction.^{22,23,24}

It has been difficult to demonstrate significant toxicity of PM in laboratory studies. Recently, studies of animals with severely impaired lungs, demonstrated high incidences of mortality and exacerbation of the pre-existing lung disease after exposure to concentrated ambient particles.²⁵ However, inhalation toxicology studies with young adult, healthy animals have not demonstrated drastic effects that could account for the observed human mortality.^{26,27}

Some of the mechanisms, relevant to the lung, by which PM induces toxic effects include aggravation of pre-existing lung or heart disease, impaired clearance or production of excess or thickened mucus, alterations of host defenses, lung inflammatory responses with changes in lung structure or cells. This project studied aged, or senescent, rats as models of elderly, susceptible individuals and assessed endpoints relevant to these mechanisms. The overall objective was to determine whether exposure to selected PM₁₀ components, alone or in mixtures, produced biological responses that could be mechanistically related to adverse PM₁₀ health effects.

The senescent rats we used had some natural debility due to age, but were otherwise in good health. We considered these rats to be more realistic models of elderly, sensitive human populations than animals that had been drastically impaired by intensive chemical or surgical treatments.

The project was performed in two Phases. Phase 1 examined the effects of specific PM components, alone and in mixtures, on mechanism-related endpoints. The primary hypotheses tested were: (1) that acid coated insoluble particles ($\leq 1 \mu$ m MMAD) would provoke deleterious responses in aged rats; and (2) that ozone (a pollutant frequently present in urban atmospheres)

would potentiate the toxicity of these particles. The data from Phase 1 were analyzed and the results presented in an Interim Report²⁸ and other publications.^{29,27} The second Phase of the study was designed with joint consultation between the investigators, ARB project management staff and Dr. Glen Cass, who served as an external reviewer and project consultant. The goal of the second Phase was to determine whether the effects of inhaled particle mixtures on mechanism-related endpoints varied as a function of particle dose. An additional hypothesis tested was (3) lung injury or responses induced by PM-related mechanisms would intensify as a function of particle concentration.

Materials and Methods

Exposures, Atmospheres and Sacrifice time

Selection of the Atmospheric Components

The concentrations and sizes of particles in the exposure atmospheres tested were selected based upon estimates of peak exposures in the South Coast Air Basin. Both C and total sulfate (SO_4^{2-}) particles are found in the 0.3-0.6 μm size range. In general, total sulfate particles are composed of sulfuric acid and ammonia neutralization products. In a previous study, we used data from the city of Azusa obtained during 1986. There is very little free sulfuric acid in ambient air in California. The dominant forms of acidic sulfate particles are most likely partially ammonia-neutralized salts, such as ammonium bisulfate (ABS), and this was the chemical species used in this study. We have reviewed the data from the 1987 SCAQS study, as well as other studies, and estimated 4 hr peak concentrations of 60 $\mu\text{g}/\text{m}^3$ ABS and 48 $\mu\text{g}/\text{m}^3$ C from the 24 hr maximum concentrations of C and total SO_4^{2-} , as follows. Solomon et al.³⁰ reported peak 24 hr elemental C concentrations of 16 $\mu\text{g}/\text{m}^3$ and peak 24 hr total sulfate concentrations of about 20 $\mu\text{g}/\text{m}^3$ for cities in the California South Coast Air Basin. Based upon data from Watson et al.³¹, we computed a peak 4 hr to peak 24 hr ratio of 3 for sulfate. For elemental C a peak 4 hr to peak 24 hr ratio of 3 was computed from the data of Turpin and Huntzicker³². The peak 4 hr concentrations were estimated by multiplying the 24 hr peak by the 4 hr/24 hr ratio. The actual C and ABS concentrations used in this study were slightly higher, 50 $\mu\text{g}/\text{m}^3$ and 70 $\mu\text{g}/\text{m}^3$, respectively. These

were selected to match those used in a prior ARB-funded exposure study so that comparisons could be made between observed effects.

Inhalation Exposure

Exposures of rats were nose-only to a continuous stream of the test atmosphere. Nose-only exposures were performed in order to prevent artifacts due to contamination of chamber air by dander, ammonia and dried excreta. Between exposures, rats lived in purified-air barrier housing and they were given purified water and fed NIH-31 Teklad Premier Laboratory Diet (Teklad, Bartonville, IL) *ad lib*. Animals were housed in wire cages over beds of rock salt that dried feces and urine and suppressed dust and ammonia production. Personnel wore gowns, hair bonnets, masks, shoe covers and surgical gloves when handling the animals to prevent the spread of pathogens to the rats. Exposure tubes were inspected daily and thoroughly machine-washed in hot soapy water after each use. Animal cages were washed and sterilized twice weekly, and quarters were cleaned daily.

Pollutant generation

Ozone was generated by passing medical grade oxygen through two corona-discharge ozonizers (Sander Ozonizer, Type III, Osterberg, Federal Republic of Germany). Sulfate aerosols were generated by nebulizing aqueous solutions of ammonium bisulfate (ABS). The aerosol was brought to Boltzmann charge equilibrium by passage through a ^{85}Kr aerosol neutralizer, and was then introduced into the exposure system purified air supply under controlled temperature and relative humidity conditions. The carbon aerosol was generated by nebulizing a suspension of Monarch 120 carbon black particles (Cabot Corp., Boston, MA). Monarch 120 consists of primary particles 75 nm in diameter, and contains less than 1% extractable organic compounds. The dry particles were suspended in water, agitated ultrasonically and nebulized with compressed air. The aerosol was dried by rapid dilution, discharged to Boltzmann equilibrium using ^{85}Kr sources and introduced into the chamber air supply. The nebulization process produced particles of carbon or ABS with 0.4 μm MMAD and a 2.0 geometric standard deviation (measured by cascade impactor). The mean was very close to the target 0.3 μm MMAD.

Pollutant Characterization

Aerosol particles were collected on preweighed and equilibrated (50% relative humidity [R.H.]) Pallflex T60A20 fluorocarbon-coated glass fiber filters (PALL Corp., Cincinnati, OH). The filters were weighed after collection to determine the total collected mass. Pallflex filters were extracted with dilute carbonate/bicarbonate buffer and the extract analyzed for SO_4^{2-} by ion chromatography.

Samples for carbon analysis were collected on acid-treated quartz fiber filters (Microquartz; Gelman, Ann Arbor, MI). Carbon was determined by combustion in oxygen in a flow-through furnace and measurement of the evolved CO_2 using a non-dispersive infrared absorption analyzer (Dasibi Model 3003, modified with a CO_2 absorption cell).

Sacrifice of rats and timing of endpoints

Phase 1 animals were euthanized immediately after their final exposure for all endpoints. The permeability measurements after the Phase 1 exposures had not shown significant atmosphere-related effects. Previous studies with younger animals had shown that the permeability change peaked 12 to 18 hr after acute exposures, and were not detectable immediately post-exposure.³³ In order to determine if the lack of permeability response immediately after the repeated exposures in Phase 1 was due to a permeability delay, we lavaged Phase 2 animals 12 hours after their last exposure. Since the Phase 2 experiments included identical purified air control, ozone and one of the particle mixture groups that had been tested in Phase 1, this protocol change permitted us to compare immediate and 12 hr permeability, inflammation and macrophage function changes as a function of time. Phase 2 animals for histological endpoints and collagen analyses were sacrificed immediately after their last exposure.

Measurements of Cellular and Non-Cellular Lung Fluid Constituents

Bronchoalveolar Lavage

The rats were anesthetized by intraperitoneal injection (i.p.) of sodium pentobarbital (50 mg/kg). The abdominal aorta was severed and a polyethylene catheter was placed and tied in the trachea. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 7 ml HEPES buffered Hanks Balanced Salt Solution

(HBSS) without Ca^{+2} or Mg^{+2} through the tracheal catheter followed by withdrawal of the fluid; at least 5 ml of lavage fluid was recovered and transferred to a 15 ml polypropylene centrifuge tube. The process of introduction and withdrawal of lavage fluid was repeated three times. The fluid from the last two lavages was pooled in a separate centrifuge tube. The tubes containing lavage fluid from each animal were centrifuged at 300 g for 10 min. The cell-free supernatant from the first lavage was analyzed for total protein and serum albumin. The cells from the two tubes were pooled for each animal.

Total Protein

A bicinchoninic acid (BCA) procedure³⁴ was used for determination of total protein in the BAL.³³ Standards were prepared by dilution from a stock solution of bovine serum albumin (BSA). Protein standards, unknown samples, and diluent used for blanks (0.10 ml each) were pipetted into appropriately labeled test tubes. Each tube then received 2 ml of BCA working reagent prepared from the kit reagents to the manufacturer's specifications (Pierce BCA Protein Assay Reagent, Pierce Chemical Co., Rockford, IL). All samples were incubated at 60°C for 30 min, then cooled. Absorbance was measured at 562 nm. Absorbances of blanks were subtracted from that for standards and unknown samples. A standard curve was prepared using known concentrations of rat albumin.

Albumin

ELISA procedures described by Schwerer et al.³⁵ and Macy et al.³⁶ were used as a guide for establishing an albumin assay in our laboratory.³³ Polystyrene non-flexible 96 well microtiter plates (Costar, Van Nuys, CA) were coated with goat anti-rat albumin antibody (Organon Teknika, Durham, NC) in carbonate buffer, pH 9.6. The plates were covered, refrigerated overnight and then washed with carbonate buffer to remove excess material or antibody. Non-specific binding was blocked by the addition of gelatin in carbonate buffer. The plates were placed in a humid chamber at room temperature for 1 to 2 hr and then washed two times with PBS-Tween 20-gelatin. A standard curve was developed using serial dilutions of a standard (15 mg/ml) rat albumin solution (Sigma Chemical Co.). Rat lavage fluid samples in PBS-Tween 20-gelatin solution were added to each well. The plates were covered and incubated in a humid chamber for 1 to 2 hrs. After washing, 100 μl of a 1:2000 dilution of peroxidase-conjugated

rabbit anti-rat albumin antibody (5.0 mg/ml) was added to each well. The plates were incubated in a humid chamber at room temperature for 1 hr. Color was developed by the addition of 100 μ l per well of citrate-phosphate buffer (pH 5.0) containing 1 mg/ml O-phenylenediamine dihydrochloride (OPD) and 1 μ l/2 ml of 30% H₂O₂. The plates were covered and incubated, in the dark, at room temperature for 20 minutes. The reaction was stopped by the addition of 50 μ l per well of 2N H₂SO₄. The plates were read at 492 nm in a plate reader (Titertek Multiscan; Salzburg, Austria).

Measurements Related to Airway Inflammation

Inflammatory Cell Infiltration (PMNs)

Cells from lavage fluid were deposited on slides using a cytocentrifuge and stained with Diff Quik (Baxter Healthcare Corp., McGaw, IL) for differential cell counts. Cells were scored as being macrophages or monocytes, lymphocytes, PMNs or “other” cells (the other category included epithelial cells, multinuclear macrophage-derived cells or cells which were not otherwise distinguishable).

Measurements Related to Acute Lung Injury and Lung Disease Processes

Collagen deposition

Total peripheral lung collagen was determined by a spectrophotometric assay for hydroxyproline, which is an amino acid unique to collagen and elastin.³⁷ Weighed samples of lung were hydrolyzed overnight at 107°C in sealed vials with 6 N HCl. After evaporation of the acid, the residue was dissolved in citrate-acetate buffer and passed through a Dowex 50x8 column (Sigma Chemical Co., St. Louis, MO) to remove carbohydrates and insoluble material. Aliquots were reacted with chloramine-T to oxidize the hydroxyproline, and then with dimethylaminobenzaldehyde to form a colored complex. Absorbance of the colored complex was measured at 450 nm. Weighed samples of lung tissue were digested in 1 N NaOH and analyzed for total protein per mg wet tissue weight using the Lowry³⁸ procedure.

Cell Proliferation in Lung Tissue

In vivo cumulative labeling index:

One hour prior to exposure, the animals were injected i.p. with 50 mg/kg BrdU (5-Bromo-2-Deoxyuridine, Sigma Chemical Corp.). Cells undergoing DNA synthesis incorporate BrdU (30 mg/ml in 0.01N sodium hydroxide), a thymidine analog, into DNA and this incorporation is indicative of cell proliferation (a marker of epithelial repair and interstitial cell response to epithelial injury). This endpoint was used to identify sites of cell turnover and numbers of cells killed in various regions of the respiratory tract.

The rats were anesthetized immediately after exposure with sodium pentobarbital (50 mg/kg i.p.). A tracheal cannula was inserted, the abdomen was opened, the liver reflected caudally, the diaphragm cut at the cardiac shadow and the lungs were collapsed to a minimum volume. After the central rib cage and sternum were removed, the lungs were removed *en bloc* and fixed at a constant pressure of 25 cm H₂O pressure with a zinc formalin fixative (Z-fix; Anatech Ltd., Battle Creek, MI) for a minimum of 24 hours. Subsequently, the lungs were removed from the fixation apparatus and stored in the same fixative until they were embedded for sectioning. Samples of gut tissue were removed and immersed in fixative for later processing.

Paraffin sections (5µm) of lung and duodenum were affixed to untreated, cleaned glass slides. The sections were deparaffinized with xylene and rehydrated through a graded series of aqueous alcohol solutions. Slides were prepared for determining morphometry changes, infiltration of inflammatory cells and cell proliferation rates by BrdU incorporation.

The BrdU incorporated into the DNA of replicating cells was detected immunocytochemically using a specific antibody and a biotin-conjugated peroxidase method. Incubating the slides with heat-inactivated normal horse serum (Sigma Chemical Co.) blocked non-specific antibody binding. Sections were then incubated overnight at 4° C with a primary mouse anti-BrdU antibody and the slides were washed with phosphate-buffered saline (PBS). The samples were then incubated for 30 minutes at 20°C with a secondary antibody (biotinylated rabbit anti-mouse IgG that that was treated for rat cross-reactivity by affinity chromatography), washed in PBS, incubated for 30 minutes at 20°C with a Vectastain ABC avidin-conjugated peroxidase reagent (Vector Labs). The samples were washed in PBS, and incubated 3-5 minutes with H₂O₂ and diaminobenzidine (DAB) as the color-forming substrate.

Counting 1000 cells per section determined labeling indices at 1000X, in terms of the number of labeled cells per 100 nuclei counted. Duodenum was used as a labeling control with each animal. Besides labeling indices, labeled cells were used in our serial section morphometry to determine total labeled cells in compartments (i.e. epithelium and interstitium) for each lobe that was examined. Counting of BrdU-labeled cells was done using a lattice system consisting of a 10 x 10 grid based on the methods described by Weibel.^{39,40} A computer-generated lattice was superimposed on a video image of the tissue section. Intersections (points) falling on structures of interest were scored separately. We used the "physical dissector" method of two serial sections to count the cells⁴¹ and the final fraction. We multiplied the number in the final fraction by the number of fractions per lobe to obtain the total number of epithelial and interstitial cells in lung lesions per lobe.⁴²

Tissue fixation for immunocytochemistry

All lung tissue used for immunocytochemical localization was preserved by perfusion fixation with zinc formalin fixative (Anatech Ltd., Battle Creek, MI) and embedded in paraffin for light microscopy.

Light microscope immunocytochemistry for BrdU

The avidin-biotin peroxidase method as outlined by Hsu et al.^{43, 44} was used. All the reagents, with the exception of the primary antibody, were supplied by Vectastain Laboratories in kit form (Vectastain ABC kit) (mouse, chicken or rabbit IgG). Controls included: (1) substitution of phosphate buffered saline (PBS), biotinylated equine anti-rabbit IgG or normal serum for the primary antibody; (2) series in which serial dilutions of the primary antibody were incubated with serial sections (culture media dilutions run from 1:2 to 1:10,000 in steps).

Selection of airway tissue for morphometry

Unique airway dissection techniques were used to isolate a minimum of 12 terminal bronchioles from the right cranial lung lobe of each rat. In brief, beginning at the lobar bronchus, the intrapulmonary airways and accompanying parenchyma were split down the long axis of the largest daughter branch or down the axial pathway of the primary airway. An attempt was made to expose as many minor daughter side-branches as possible. From the dissected

specimen, the lobe was dehydrated and embedded dissected airway down in paraffin. Sections that were 30 μm and 5 μm -thick were cut for BrdU and Hematoxylin/Eosin (H & E) staining, respectively. Twelve to twenty terminal bronchioles were visible in each section and each one was given a sequential number beginning at the hilus and ending at the pleural surface. Four terminal bronchioles were selected from each section using stratified sampling with a random start within the first interval (i.e., if there were 16 terminal bronchioles, then the random number was selected from 1-4 for the first selection and each subsequent selection was the initial number +4). This method of sampling provided for unbiased selection of terminal bronchioles independent of size or location.

Morphometry of airways

Computer software developed by Dr. Hyde, “Sterology Toolbox,” was used for morphometric analyses. The number of BrdU labeled epithelial cells was estimated using the optical “dissector” technique and counting BrdU labeled cells in 30 μm sections. Every field of each sectioned terminal bronchiole was counted. Labeled cells in the top focal plane of the section were not counted, but all the labeled cells at deeper focal planes in the section were counted. Since we knew the area of each sampling point of the test system hitting epithelium or interstitium, the number of labeled cells per unit area of epithelium or interstitium of a terminal bronchiole could be calculated directly. The formula divided the total number of labeled epithelial or interstitial cells (N count) by the number of points that hit epithelium (P epi) or interstitium (P int) and that ratio was divided by the area per point in mm^2 (A_p) to calculate the number of labeled epithelial or interstitial cells per unit area (N_a) (mm^2) as follows:

$$N_a(\text{epi}) = (N \text{ epi} / P \text{ epi}) / A_p \dots \dots \dots (1)$$

$$N_a(\text{int}) = (N \text{ int} / P \text{ int}) / A_p \dots \dots \dots (2)$$

These values were multiplied by the thickness of the focal plane to estimate volumes.

Morphometry of lung lobes

Morphometric measurements were used to estimate the volume of lung lobes, the cumulative volume of inflammatory and to estimate the extent of fibrotic lesion within each lung lobe, and the components of the lesion, both interstitial and epithelial, within each lobe.⁴⁵ The interstitial lesion components include inflammatory cells, fibroblasts and smooth muscle cells,

endothelial cells associated with vessels, and extracellular fibers and amorphous matrix. The epithelial lesion components include epithelial cells (all types including necrotic and degenerating cells) and migratory inflammatory cells. To estimate the volume of each lung lobe, about ten samples were taken from the 30 μ sections (selected systematically), by systematically subdividing the 30 μ sections into smaller and smaller fractions until a final fraction was obtained. The lobe volume was estimated in the final fraction volume by multiplying the volumes of the individual blocks of the final fraction and the number of fractions taken from the lobe. The volumes of the individual blocks (usually six) were determined as the product of the block areas and their heights, and the sampling fractions were recorded when the lobe was cut during sampling.^{46,47} The advantage of this approach was that the volume obtained was the fixed, embedded and sectioned volume. Thus, when the number of fibroblasts within lung lesions was counted in the final fraction (the final six sampled blocks), there was no bias created by multiplying by the sampling fractions, as there would be if we measured lung volume by fluid displacement which measures the volume of the fixed lung. The "physical dissector" method was used to determine the number of epithelial and interstitial cells (including those labeled by BrdU) per mm³ in the final fraction of two serial sections.⁴¹ The number in the final fraction was multiplied by the number of fractions per lobe to obtain the total number of epithelial and interstitial cells in lung lesions per lobe.^{47,48}

Excised lung gas volume

Excised lung gas volume (ELGV) was measured from rats (n = 10 per group) immediately following sacrifice. Following deep anesthesia, the abdominal aorta was exposed and severed to exsanguinate the rat. The diaphragm was then cut allowing the lungs to collapse and retain the volume of trapped gas behind the closed airways as described below and elsewhere.^{49,50,51,52,53,54,55} The trachea was ligated, and the lungs and trachea were removed. They were tied to a weight and suspended under saline in a vessel attached to a balance. The volume of the trapped gas was then measured as buoyant force of the lungs on the tared vessel and ballast weight immersed in saline.

Following measurement of ELGV, the lungs were removed from the apparatus, blotted, and the wet mass of the lung lobes was measured. The lobes were then dried to constant mass at 70°C for measurement of dry mass, and wet to dry mass ratio was calculated.

Measurements Related to Respiratory Infection

Fc Receptor Binding

Macrophages (10^5 cells) isolated from lavage fluid, were incubated in Tissue-Tek chambers in HBSS with Ca^{++} and Mg^{++} for 60 min. Nonadherent cells were removed by washing with HBSS. For assay of Fc receptor binding, adherent macrophages, were incubated with rat anti-sheep red blood cell antibody,^{56,57} excess antibody was removed by washing with HBSS, and the cells were incubated with sheep red blood cells (SRBC). Excess SRBC were removed by washing with HBSS, and the macrophages were examined using an inverted phase contrast microscope. The number of rosettes, or macrophages attached to 3 or more SRBC, was scored as positive. Control preparations, using macrophages, unstimulated with antibody, were scored as described above to correct the number of rosettes observed for non-specific binding.

Phagocytosis

For phagocytosis assays, 5×10^5 macrophages were incubated at 37°C in suspension with 10^8 fluorescent polystyrene latex particles (1 μm diameter) for 60 min. The suspensions were agitated and 1 mL aliquots were withdrawn and transferred to a cytocentrifuge. The samples were centrifuged onto labeled microscope slides and fixed in situ with methanol. The slides were stained with Dif-Quik (Baxter) and treated with xylene to quench the fluorescence of unengulfed polystyrene particles. Cells containing 2 or more engulfed particles were scored as positive. The number of positive macrophages and the numbers of particles ingested by positive macrophages were determined.²⁷

Respiratory Burst Activity

Integrated superoxide production during respiratory burst activity after stimulation with opsonized zymosan (Sigma Chemical, St. Louis, MO) were measured in the presence and absence of superoxide dismutase (SOD) as follows. Lung lavage cells (2×10^5) were added to luminometer cuvettes (LKB Pharmacia) in 1 ml HHBS. Samples were incubated at 37°C in 5% CO_2 for 90 min and non-adherent cells were removed by gentle washing. The medium was replaced with RPMI 1640 supplemented with 10 mM glucose. SOD-inhibitable superoxide production was determined by lucigenin-amplified chemiluminescence using an LKB-Pharmacia Model 1251 Luminometer. Chemiluminescence measurements were made beginning immediately after the addition of 200 mM bis N Methylacridinium Nitrate (lucigenin, Sigma),

with or without stimulating agent (50 ng/mL opsonized zymosan). Measurements were continued until readings returned to near baseline levels (typically 30 min). Duplicate cuvettes with SOD-treated samples were used to correct readings to yield SOD-inhibitable chemiluminescence readings.

Measurements Related to Chronic Bronchitis

Mucus glycoprotein by ELISA

Serial dilutions of BAL samples were plated, in duplicate, in 96 well microtiter plates previously activated with 0.1% glutaraldehyde (in 0.1 M sodium carbonate, pH 9.0) and poly-L-lysine (in 0.05 M Na bicarbonate). Wells containing dialyzed sputum glycoproteins excluded from Sepharose 4B (void volume, V_0 fraction) from humans with cystic fibrosis served as positive controls on each plate. The plates were incubated overnight at 40°C. The next morning, plates were blocked with 1% normal goat serum/PBS/Tween (GPT) before being incubated for 2 h at room temperature with antibody 10G5 (hybridoma supernatant diluted 1:10) in GPT. Plates were washed with GPT and incubated with goat anti-mouse IgG β -galactosidase conjugate at a 1:200 dilution in PBS containing 1.5 mM $MgCl_2$, 0.05% Na Azide, 2 mM mercaptoethanol for another 2 h at room temperature. After washing, a β -galactosidase substrate (p-nitrophenyl-b-galactophyranosidase: 1 mg/ml) was applied to the plate. Absorbance values were read at 405 nm after 15 m at 37°C. The absorbance values produced by BAL supernatants from pollutant-exposed and air-exposed rats were compared to determine the effect of pollutant exposure on basal mucin secretion (i.e., secreted mucin glycoprotein) in the pulmonary airways. Absolute values were obtained by reference to a standard curve constructed using hyaluronidase-treated mucin V_0 fraction material. These assays were performed in the laboratory of Dr. Carol Basbaum, UCSF.

Statistical Analyses

The morphometric analyses evaluate sample mean values to within $\pm 10\%$ using a 95% confidence interval with the Student t distribution.⁴⁸ Multiple comparison tests were used to assess significant differences among groups and time points.⁵⁸ Data transforms or nonparametric statistical methods were used to analyze variables that were not normally distributed and for comparing groups that had unequal variances. Two-tailed tests with $\alpha \leq 0.05$ were used to establish statistical significance.

Results

Phase 1. Toxicity of PM Components

Ammonium bisulfate (ABS) and elemental carbon were the particulate components selected for this study. These components represent substantial fractions of the ambient fine acidic particles. Acid compounds were formed in the atmosphere by the heterogeneous nucleation and oxidation of gaseous precursors (SO_2 and NO_2). The resulting acid compounds can form coatings on existing ambient particles (e.g. diesel soot).⁵⁹ Acid-coated particles have been demonstrated to be more toxic than the equivalent doses of acids presented as uncoated particles, and therefore are important to study.⁶⁰ Photochemical processes are important for the formation of acid particles and also for ozone (O_3) in ambient atmospheres, especially in southern California. Thus O_3 is often coexistent with acid particles. For this reason, particle-containing atmospheres in this study were tested for toxicity with and without O_3 . The study design and the endpoints assessed are summarized in Table 1. The Phase 1 objective was to compare effects on mechanism-related endpoints of individual components with those of mixtures.

Exposure Parameters

Aged (22 to 24 months), barrier reared F344N-Nia rats were obtained from colonies managed under contract to the National Institute on Aging (Bethesda, MD). The rats were shipped in filter-equipped boxes and were housed in laminar flow isolation units supplied with filtered air. They were allowed access to food and water *ad lib*. The rats were randomly assigned to treatment groups and nose-only exposed, 4 hours per day, 3 consecutive days per week, for 4 weeks, to one of six atmospheres: (1) purified air; (2) C, $50 \mu\text{g}/\text{m}^3$, $0.3 \mu\text{m}$ MMAD; (3) ABS, $70 \mu\text{g}/\text{m}^3$, $0.3 \mu\text{m}$ MMAD; (4) O_3 , 0.2 ppm; (5) ABS + C; and (6) ABS + C + O_3 . The concentrations of components and particle size data are summarized in Table 2. The atmospheres were well controlled and the size/concentration values met the stated specifications.

Ten animals from each group were assessed for histopathology endpoints and 12 from each group were assessed for macrophage and permeability-related endpoints. Between exposures, rats were returned to the purified air-barrier environment and given access to clean water and dry laboratory chow *ad lib*.

Table 1 Atmospheres and Endpoints Evaluated in Phase 1

Group	Atmosphere	Conc.	Exposure	Endpoints
1	Purified Air		4 hr/d, 3d/wk, 4 wk	Collagen Deposition
2	Carbon (0.3 μ m)	50 μ g/m ³	"	Cell Proliferation
3	Carbon + ABS	50 μ g/m ³ 70 μ g/m ³	"	Lung morphometry Epithelial Permeability
4	O ₃	0.2 ppm	"	Macrophage Phagocytic Activity
5	Carbon + O ₃	50 μ g/m ³ 0.2 ppm	"	Fc Receptor Binding
6	Carbon + ABS + O ₃	50 μ g/m ³ 70 μ g/m ³ 0.2 ppm	"	Respiratory Burst Activity Excised Lung Gas Volume Mucus Glycoprotein

Table 2 Atmosphere Component Concentrations and Particle Sizes Measured During Phase 1 Exposures

Group	Atmosphere	Particle Size ^a (MMAD; GSD)	Actual Concentration Mean, $\mu\text{g}/\text{m}^3 \pm \text{SD}$	Relative Humidity ^b (%)
1	Purified Air	NA ^c	NA	59.1 \pm 3.0
2	Carbon	0.29 μm ; 1.9	46.27 \pm 12.64	
3	Carbon + ABS	0.38 μm ; 2.4	55.80 \pm 25.34 61.03 \pm 20.35	
4	O ₃	NA	0.209 \pm 0.019	
5	Carbon + O ₃	0.30 μm ; 2.0	47.34 \pm 13.00 0.205 \pm 0.008	
6	Carbon + ABS + O ₃	0.38 μm ; 2.4	53.96 \pm 25.51 66.01 \pm 21.58 0.206 \pm 0.010	

^a The particles size values for Carbon represent pooled averages of 3 samples each for atmospheres 2 and 5. The values for Carbon + ABS represent pooled averages for atmospheres 3 and 6.

^b Relative humidity was measured in the purified air chamber.

^c NA – Not Applicable

Exposure-related biological responses

Lung Injury

Cell Replication/BrdU Labeling

We identified the location of cell replication, and the cell types involved in this replication, using a DNA-specific tracer (BrdU). Both interstitial and epithelial cells were examined. Increased labeling of lung cells occurred following all pollutant exposures, relative to purified air (Figure 1). However, group mean value differences were significant only in the group of rats exposed to the atmosphere containing a combination of ABS, O₃ and C, compared to the purified air controls. Although the epithelial cells are most likely impacted by inhaled particles first, the adjacent interstitial cells have greater replication rates. The Phase 2 study examined the hypothesis that the sensitivity of the lung cells varied as a function of dose.

Lung Collagen

Collagen was measured in samples of lung tissue obtained from the rats immediately post-exposure. The data, which are shown in Figure 2, show a significant decrease in the collagen content of the lungs of rats exposed to the O₃ + C + ABS mixture, and smaller decreases in lungs of rats exposed to the other pollutants.

Morphometric Assessment of Lesions

Lungs from all groups were assessed for the presence of fibrotic lesions. No evidence of such lesions was observed, although the lungs were scanned systematically by Dr. Hyde.

Figure 1 BrdU Labeling of Interstitial and Epithelial Cells. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O_3 = 0.2 ppm Ozone. Values are Mean \pm SE.

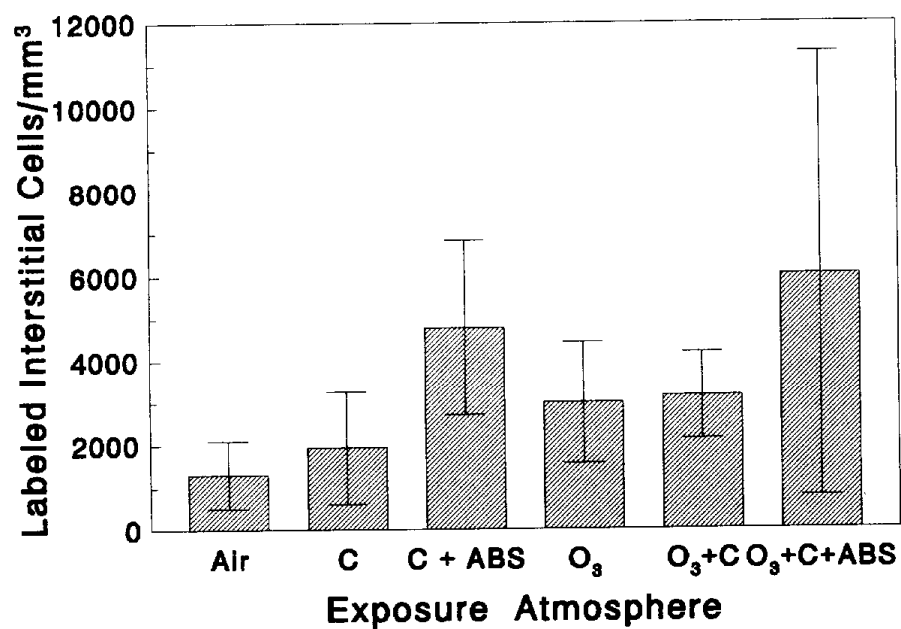
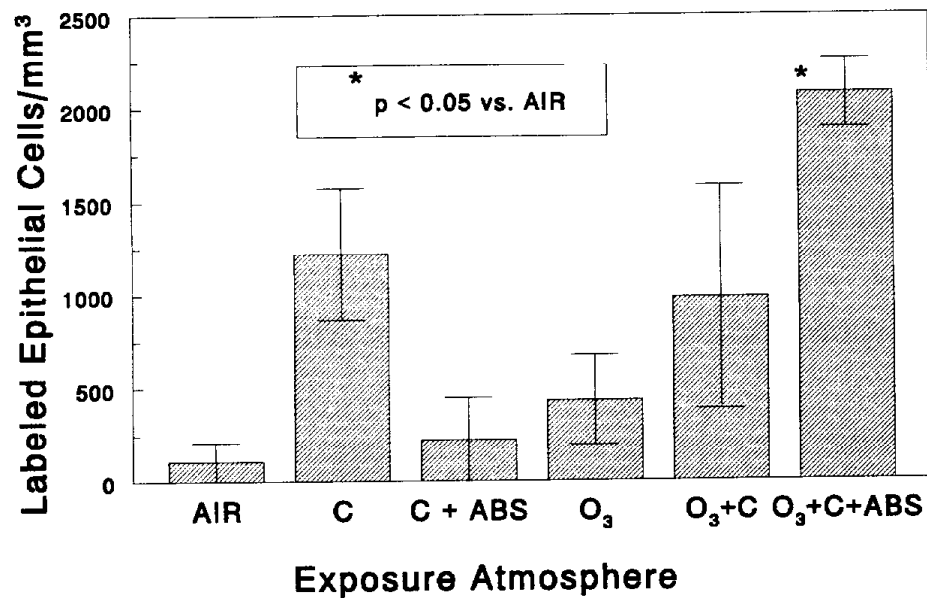
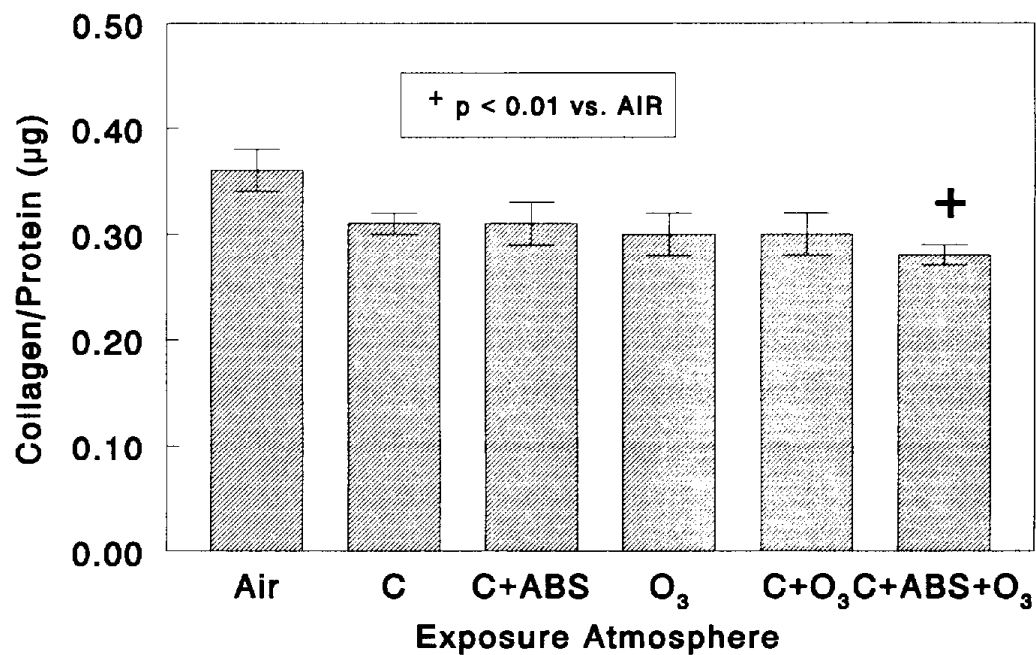


Figure 2 Collagen in Lung Tissue After Exposure to PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.



Macrophage Function

Cells recovered from rats by bronchoalveolar lavage had, on the average, viabilities greater than 94%. There were no exposure-related increases in numbers of cells recovered or the percentages of polymorphonuclear (PMN) cells. These data are summarized in Table 3. Macrophages represented between 95% and 100% of the recovered cells. Macrophages from rats exposed to PM10 components exhibited slightly, but not significantly, depressed ability to attack antigenic material (SRBC) via Fc receptor-mediated processes (Figure 3). The production of superoxide during respiratory burst activity following stimulation with opsonized zymosan (Figure 4) was increased by exposures to the particle-containing atmospheres. The superoxide production following exposures to the ABS + C + O₃ mixture was significantly greater than that observed for O₃ alone. Exposures to all three pollutants at low concentrations produced a trend toward increased production of superoxide by macrophages, but the changes were not statistically significantly different from the purified air group values. We found that phagocytic activity was slightly increased after particle exposures and slightly depressed after ozone exposure (Figure 5). However, combined particle plus ozone exposures resulted in significantly ($p \leq 0.05$) increased phagocytic activity. These results are consistent with those reported by Jakab and Hemenway,⁶¹ even though the present study was performed at relatively low concentrations (0.2 ppm O₃ and 70 µg/m³ carbon), while that of Jakab and Hemenway was performed with 1.5 ppm O₃ and 10,000 µg/m³ carbon concentrations.

Table 3 Characteristics of Cells Recovered From Bronchoalveolar Lavage Fluid. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean ± SE.

Group	Atmosphere	Yield (10 ⁶ Cells)	Viability (%)	PMNs (%)
1	Purified Air	3.6 ± 0.2	95.5 ± 1.6	0.67 ± 0.23
2	O ₃	3.4 ± 0.3	94.1 ± 2.5	0.77 ± 0.33
3	C	3.8 ± 0.3	97.3 ± 0.6	0.53 ± 0.14
4	C + ABS	3.5 ± 0.2	94.2 ± 2.0	0.59 ± 0.19
5	C + O ₃	4.3 ± 0.5	98.0 ± 0.5	0.82 ± 0.27
6	C + ABS + O ₃	3.6 ± 2.4	95.8 ± 0.9	0.77 ± 0.19

Figure 3 Fc Receptor Binding Following Exposure to PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.

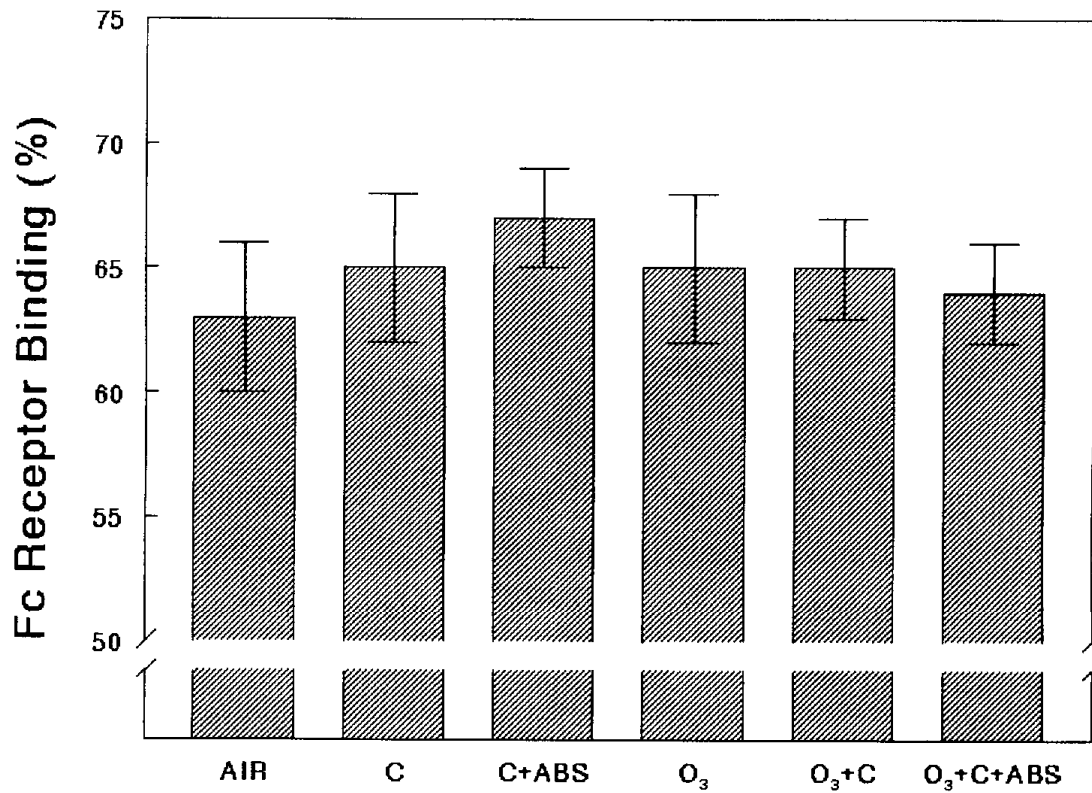


Figure 4. Respiratory Burst Activity Immediately After Exposure to PM Components.
Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$
Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm
SE.

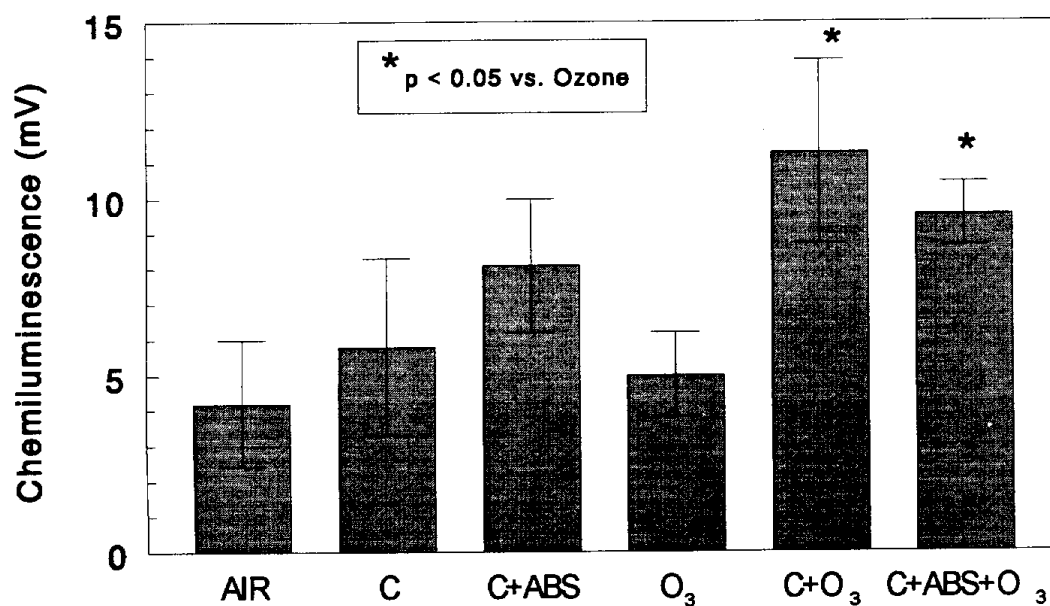
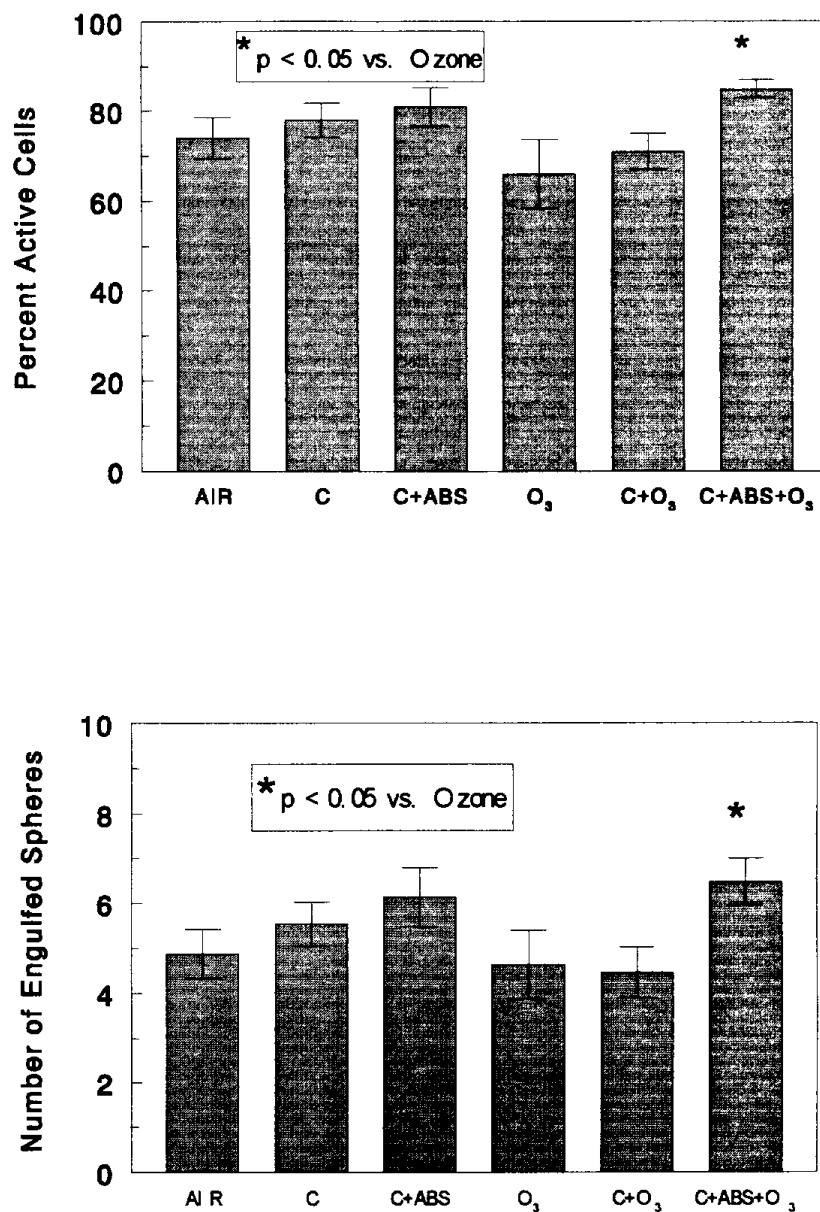


Figure 5 Macrophage Phagocytic Activity After Exposure to PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.



Permeability and Airway Inflammation

Bronchoalveolar lavage fluid (BAL) was analyzed for albumin and total protein as indicators of epithelial disruption and increased mucosal permeability. The results are shown in Figure 6. None of the atmospheres significantly increased BAL concentrations of either total protein or albumin, in the presence or absence of O₃. This study examined BAL collected immediately post-exposure. Phase 2 studies examined the hypothesis that permeability changes following repeated PM exposures would be observed 12 to 18 hr post-exposure.

Airway inflammation and excess mucus have been observed in the bronchial biopsies of patients with mild bronchitis and asthma. Inflammatory cells have also been noted in sputum and bronchoalveolar lavage fluids of asthmatics. Inflammation has also been recognized as a principal component of the airway response to air pollutants, such as O₃, in animals and humans. However, exposure to particle-containing atmospheres, at concentrations selected for this study did not have a significant effect on the inflammatory (PMN) cell population recovered in BAL (Table 3). In general, O₃-containing atmospheres resulted in a slightly, but not significantly higher fraction of PMNs in the BAL. Mucus glycoprotein concentrations (Figure 7) showed no significant exposure-related pattern.

Excised Lung Gas Volume (ELGV)

The volume of gas trapped in excised lungs from PM-exposed and control rats was assessed as an indication of functional changes that could be associated with changes in lung structure. Increased ELGV would be an indication of an adverse response. No atmosphere-related increases in ELGV were observed (Figure 8).

Figure 6 Epithelial Permeability Immediately After Exposure to PM Components.
Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.

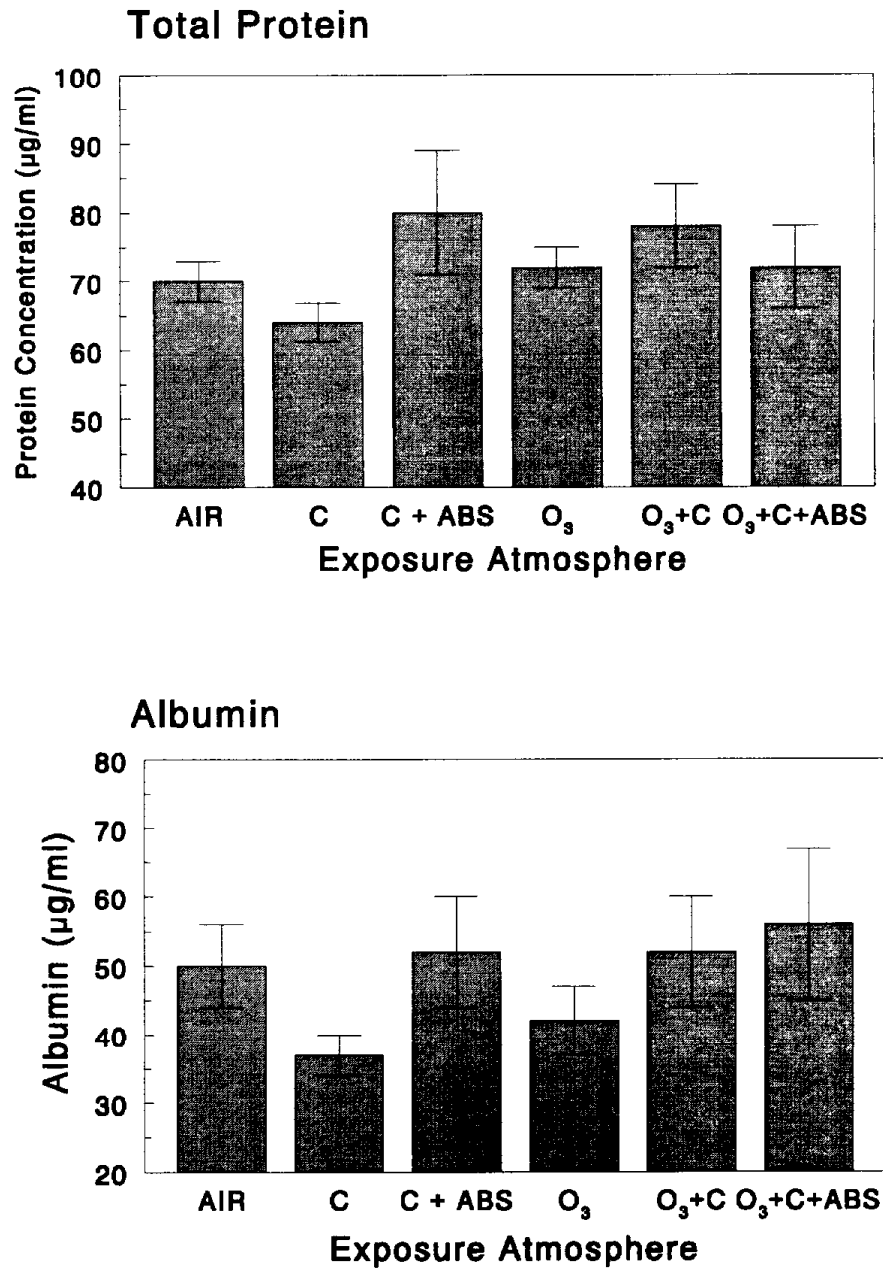


Figure 7 Mucus Glycoprotein in Bronchoalveolar Lavage Fluid After Exposure to PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.

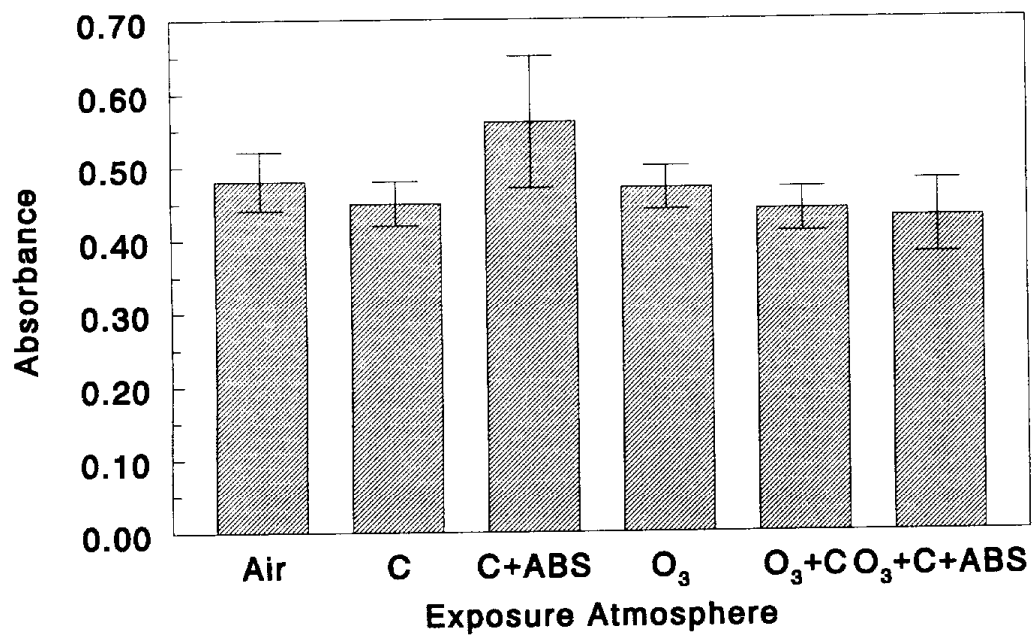
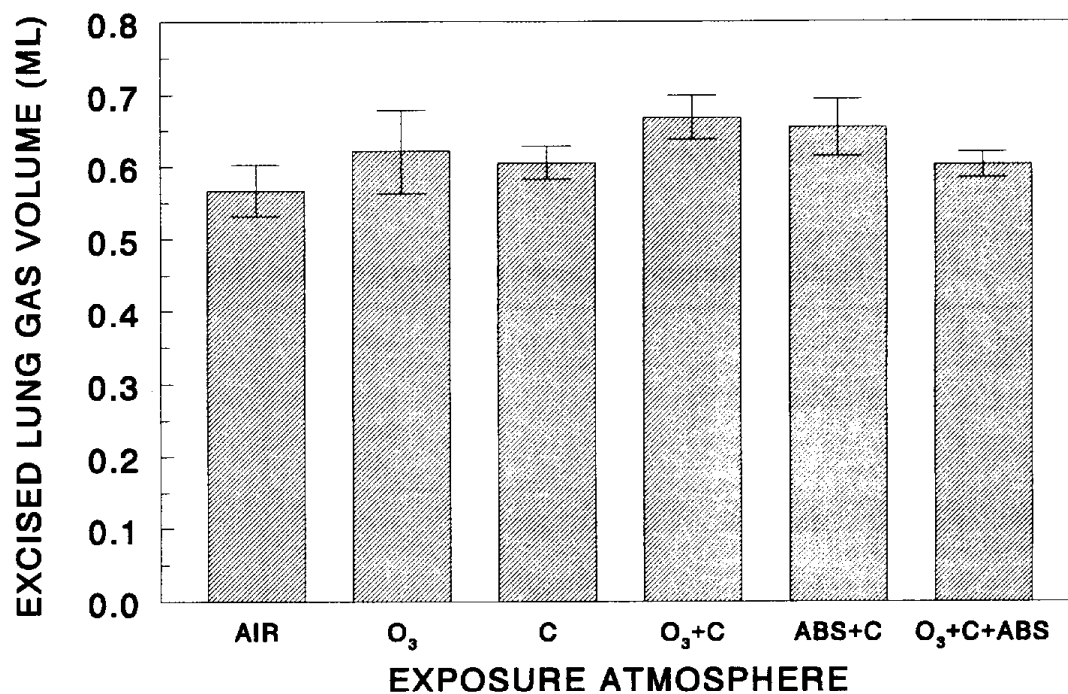


Figure 8 Excised Lung Gas Volume in Rats After Exposure to PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.



Phase 2. Dependence of Toxicity on Dose

The Phase 2 study was designed to build upon the Phase 1 findings and to assess the relationships between exposure concentrations and biological responses. The Phase 1 results indicated that the C+ABS+O₃ mixture was more toxic than any of the individual components or two-component mixtures. This mixture is representative of ambient fine particle exposures in California (Chow et al., 1992), and during episodes people are exposed to C, sulfates and O₃ at, or near, the same time. The study design and endpoints assessed are shown in Table 4.

Table 4 Atmospheres and Endpoints Tested in Phase 2

Group	Atmosphere	Conc.	Exposure	Endpoints
1	Purified Air		4 hr/d, 3d/wk, 4 wk	Collagen Deposition, Cell Proliferation, Lung morphometry, Epithelial Permeability, Macrophage Phagocytic Activity, Fc Receptor Binding, Respiratory Burst Activity, Excised Lung Gas Volume, Mucus Glycoprotein
2	Ozone	0.2 ppm	"	
3	Carbon + ABS (0.3 mm) + Ozone	50 mg/m ³ 70 mg/m ³ + 0.2 ppm	"	
4	Carbon + ABS + O ₃	100 mg/m ³ 140 mg/m ³ 0.2 ppm	"	

Exposure Parameters

Aged (22 to 24 months) barrier reared and maintained F344N-NIA rats were obtained from colonies managed under contract to the National Institute on Aging (Bethesda, MD). Rats were shipped to our laboratory in filter-equipped boxes. The rats were housed in laminar flow isolation units supplied with filtered air, and allowed access to food and water *ad lib*. The rats were randomly assigned to treatment groups. The rats were nose-only exposed, 4 hours per day, 3 consecutive days per week, for 4 weeks, to one of four atmospheres: (1) purified air; (2) O₃, 0.2 ppm; (3) C, 50 µg/m³, 0.3 µm MMAD + ABS, 70 µg/m³, 0.3 µm MMAD + O₃, 0.2 ppm; and (4) C, 100 µg/m³, 0.3 µm MMAD + ABS, 140 µg/m³, 0.3 µm MMAD + O₃. In this experiment, the concentrations of the particulate species were doubled while the O₃ concentration was held constant. Ten animals from each group were assessed for histopathology endpoints and 12 from each group were assessed for macrophage and permeability-related endpoints. Between exposures, rats were returned to the purified air-barrier environment and given access to clean water and dry laboratory chow *ad lib*. The concentrations of atmospheric constituents and particle size characteristics are summarized in Table 5. The atmospheres were well controlled and well matched to the target size and concentration values specified in Table 4. The concentrations of the components of atmospheres 2 and 3 were nearly identical to those of Phase 1 atmospheres 2 and 6 (Table 2).

Table 5 Concentrations and Particle Sizes of Constituents of Phase 2 Atmospheres

(Mean \pm SE)

Group	Atmosphere	Particle Size MMAD (μm); GSD	Actual Concentration	Relative Humidity
1	Purified Air	NA ^a	NA	59.88 \pm 0.84
2	O ₃	NA	0.198 \pm 0.004	
3	Carbon + ABS + O ₃	0.30 μm ; 2.5	51.35 \pm 12.15 76.25 \pm 18.36 0.194 \pm 0.004	
4	Carbon + ABS + O ₃	0.29 μm ; 2.3	92.35 \pm 18.51 136.29 \pm 27.61 0.197 \pm 0.003	

^aNot Applicable

Exposure-related Biological Responses

Cell Replication/BrdU Labeling

Labeling of DNA in lung epithelial and interstitial cells undergoing replication at the end of the 4-week exposure period was determined as previously described. The numbers of labeled cells per unit tissue volume were increased at both particle dose levels compared to those in tissue from either purified air or O₃-exposed rats. As summarized in Table 6, O₃ modestly (20 to 40%) increased cell replication rates in interstitial and epithelial cells, but cell replication rates in particle-exposed rat's lungs were increased by between 250 to 340%, relative to purified air. The results, which are shown in Figure 9 and Figure 10 for epithelial and interstitial cells, respectively, suggest that mechanisms of injury and cell replication may vary as a function of particle dose. The data demonstrate that more cell replication was induced by particle mixture exposures in the interstitial region as compared to the epithelial region relative to the baseline turnover rate (*i.e.* that seen after purified air exposures). Thus, ratios of labeled

interstitial/labeled epithelial cells are slightly, but not significantly, greater in lungs from particle-exposed rats than in those from either air or O₃-exposed rats (Table 6).

Morphometric evaluations of the lung were performed and there was no evidence of histopathologic lesions. Nor were there significant atmosphere-related differences in lung collagen content. Measurements of excised lung gas volumes did not evidence any atmosphere-related changes.

Table 6 Labeling of Replicating Epithelial and Interstitial Cells Relative to Controls As a Function of Concentration. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 µg/m³ Carbon; ABS = 140 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean ± SE.

Group	Per Unit Tissue Volume		% of Purified Air		Ratio (Int/Epi)
	Epithelial	Interstitial	Epithelial	Interstitial	
Air	2.7 ± 0.5	4.2 ± 0.9	100 ± 20	100 ± 20	1.5 ± 0.1
Ozone	3.7 ± 1.5	5.3 ± 2.0	140 ± 50	120 ± 50	2.1 ± 0.4
Low Mix	8.4 ± 3.1	14.6 ± 3.7	310 ± 110	340 ± 90	2.6 ± 0.7
High Mix	5.6 ± 1.6	12.5 ± 4.5	200 ± 60	290 ± 110	2.5 ± 0.8

Figure 9 Labeling of Epithelial Cells After Exposure to Low and High Concentrations of Particles, Compared to Purified Air and O₃ Alone. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 µg/m³ Carbon; ABS = 140 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean ± SE.

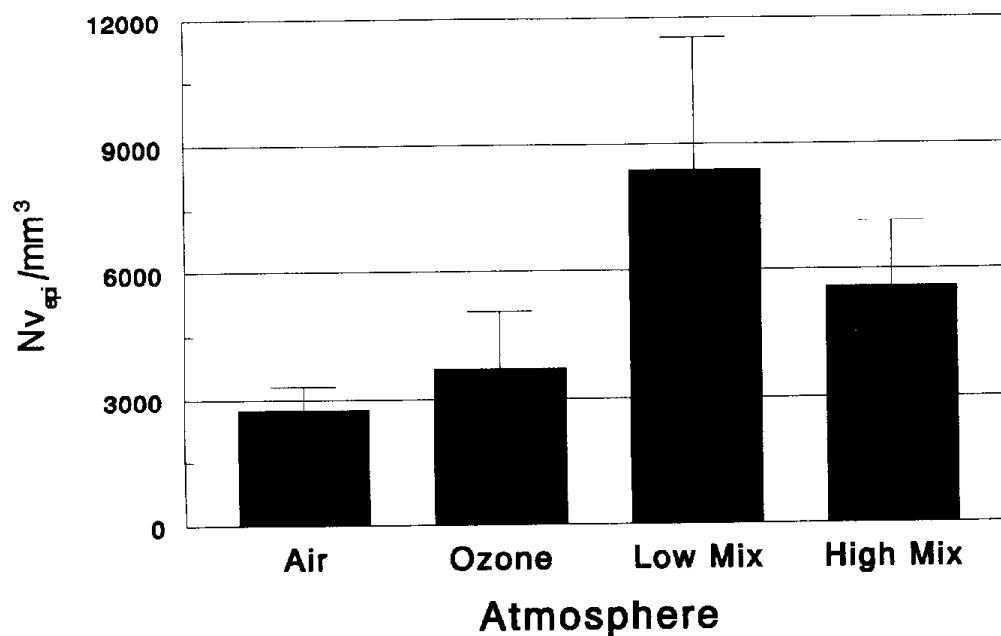
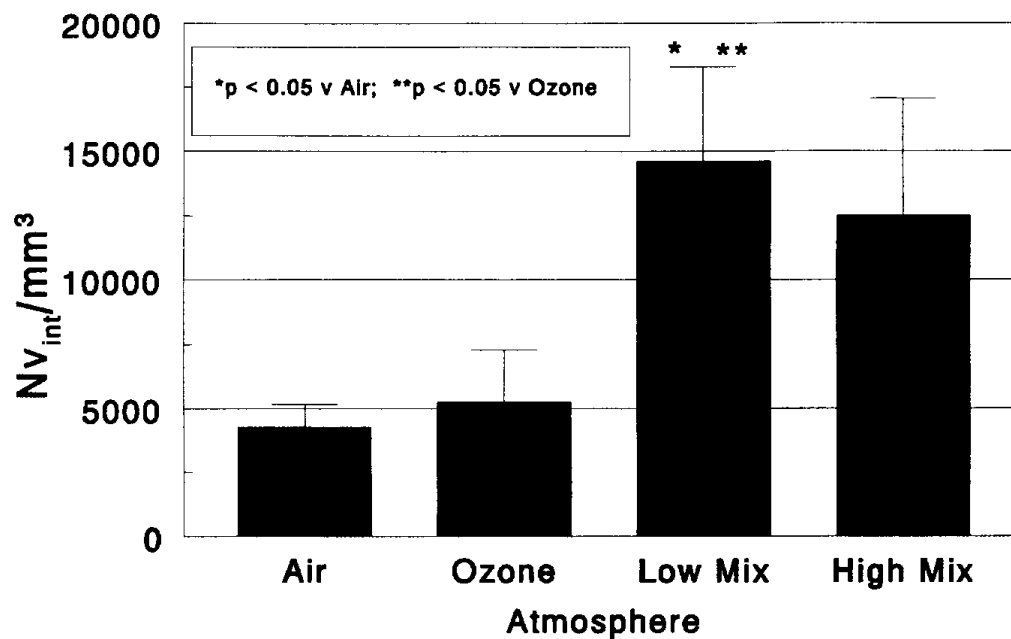


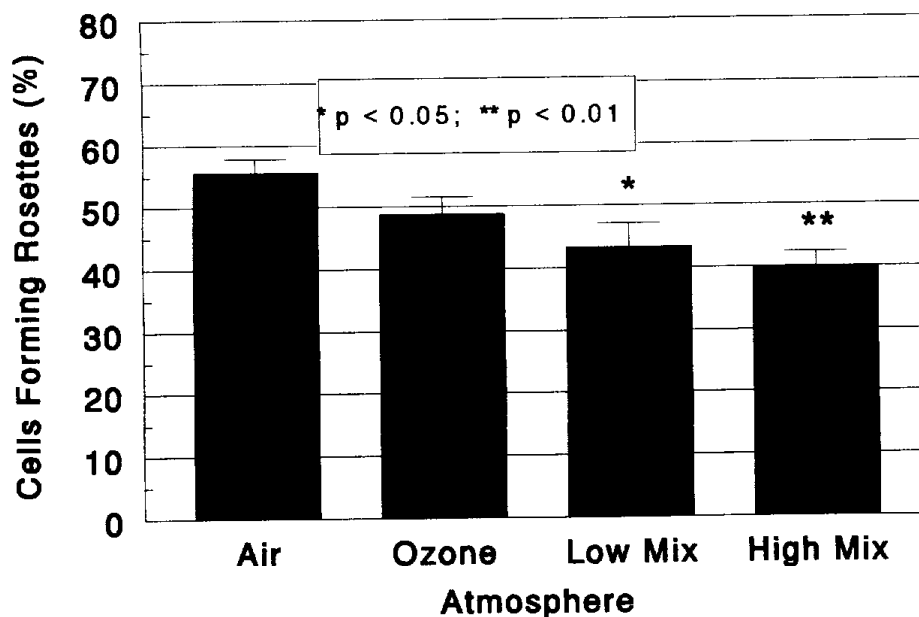
Figure 10. Labeling of Interstitial Cells After Exposure to Low and High Concentrations of Particles, Compared to Purified Air and O₃ Alone. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 140 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean \pm SE.



Macrophage Function

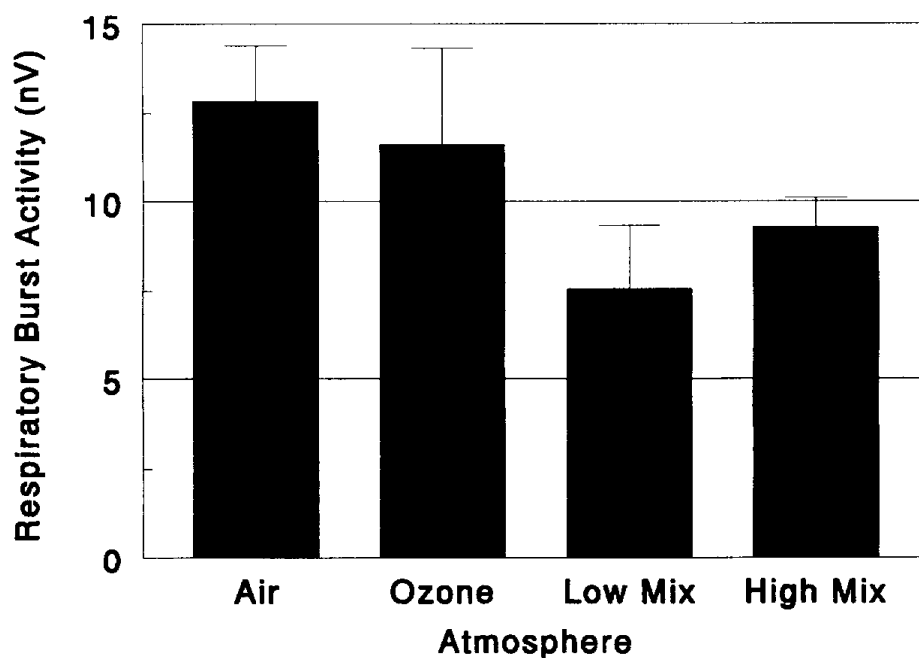
Greater than 90% of the cells recovered from these senescent rats by bronchoalveolar lavage were viable. There were no exposure-related increases in numbers or percentages of lymphocytes or polymorphonuclear (PMN) cells and macrophages represented between 95% and 100% of the recovered cells. Macrophages from rats exposed to both the high and low concentrations of the PM10 component + O₃ mixtures exhibited significantly depressed ability to attack antigenic material (SRBC) via Fc receptor-mediated processes (Figure 11). Phagocytic activities for non-specific particles (polystyrene microspheres) did not, however, evidence any significant atmosphere-related changes.

Figure 11 Macrophage Fc Receptor Binding After Exposure to Low and High Concentrations of PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 µg/m³ Carbon; ABS = 140 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean ± SE.



The production of superoxide during respiratory burst activity following stimulation with opsonized zymosan (Figure 12) was depressed by exposures to both concentrations of the particle-containing atmospheres relative to either purified air or O₃. Comparisons were made between macrophage function endpoints in rats euthanized immediately post-exposure and rats euthanized 12 hr post-exposure. The results suggest that at the end of particle exposure macrophages are activated, but when macrophages are recovered 12 hr post-exposure, they are functionally depressed.

Figure 12 Macrophage Respiratory Burst Activity After Exposure to Low and High Concentrations of PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 µg/m³ Carbon; ABS = 140 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone. Values are Mean ± SE.



Permeability and Airway Inflammation

Bronchoalveolar lavage fluid (BAL) collected 12 hr post-exposure was analyzed for albumin and total protein as indicators of epithelial disruption and increased mucosal permeability. The results are shown in Figure 13. Phase 1 of this study showed small, but not significant, increases in BAL concentrations of total protein and albumin (Figure 4) immediately following exposure to PM10 components. The Phase 2 study shows that permeability was slightly increased by ozone and significantly increased by exposure to the low concentration particle + O₃ mixture when lung epithelial permeability is measured 12 hr post-exposure.

Airway inflammation was also examined. Exposure to both high and low concentrations of a particle-containing atmosphere did not have a significant effect on cell viability, cell yield or the inflammatory (PMN) cell population recovered in BAL (Table 7). In general, O₃-containing atmospheres resulted in a slightly, but not significantly higher fraction of PMNs in the BAL. Mucus glycoprotein concentrations (Figure 14) showed a pattern of responses that was similar to that seen for morphometric, permeability and macrophage endpoints. The particle-containing atmospheres tended to have greater effects than O₃ alone, but the lower concentration was more effective than the higher concentration in provoking these changes. It should however be noted that, for most endpoints, the group mean values for the high and low concentration exposures were not significantly different from each other for any of the endpoints tested, so questions of potency cannot be answered unequivocally.

Table 7 Cells Recovered in Bronchoalveolar Lavage Fluid From Rats Exposed to Low and High Concentrations of PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 µg/m³ Carbon; ABS = 70 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 µg/m³ Carbon; ABS = 140 µg/m³ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone.

Group	Percent ± SE			
	Macrophages	PMNs	Lymphocytes	Other
Air	93.4 ± 0.3	0	6.2 ± 0.3	0.31 ± 0.04
Ozone	93.7 ± 0.4	0.10 ± 0.02	5.3 ± 0.4	0.88 ± 0.07
Low Mixture	93.1 ± 0.4	0.09 ± 0.01	6.1 ± 0.3	0.73 ± 0.05
High Mixture	94.6 ± 0.4	0.14 ± 0.03	5.0 ± 0.4	0.42 ± 0.04

Figure 13 Epithelial Permeability 12 hr Post-Exposure to Low and High Concentrations of PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 140 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone.

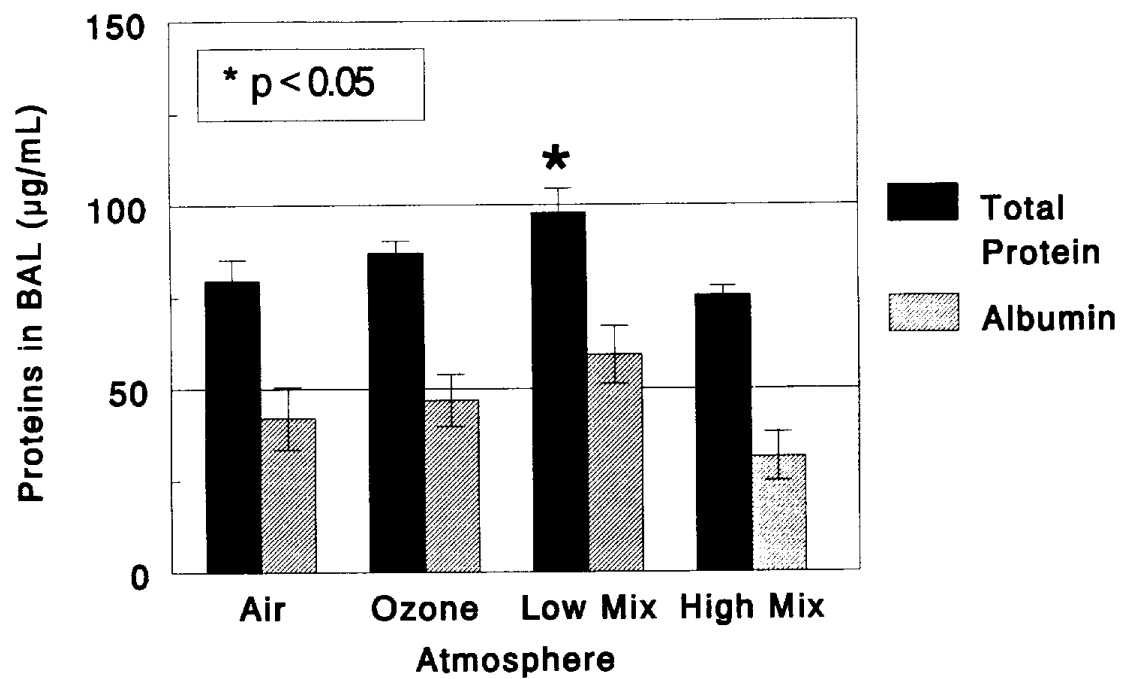
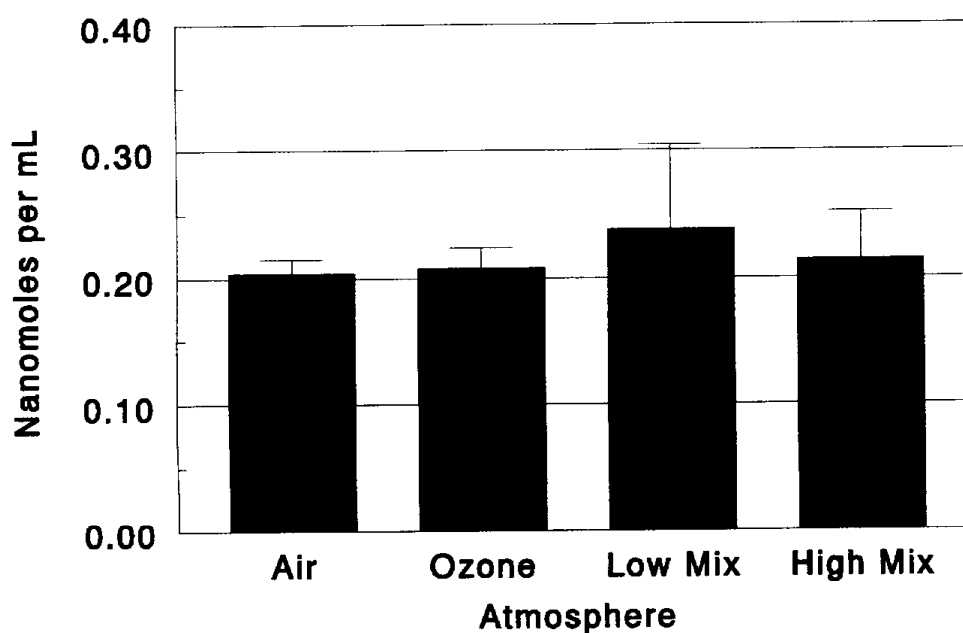


Figure 14 Mucus Glycoprotein in Bronchoalveolar Lavage Fluid 12 hr After Exposure to Low and High Concentrations of PM Components. Nominal exposure atmosphere concentrations were: AIR = purified air; Low Mix contained C = 50 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 70 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone, High Mix contained C = 100 $\mu\text{g}/\text{m}^3$ Carbon; ABS = 140 $\mu\text{g}/\text{m}^3$ Ammonium Bisulfate; O₃ = 0.2 ppm Ozone.



Discussion

The relationship between environmental PM₁₀ exposure and acute mortality is of continuing concern. Currently, much attention is focused on the fraction of PM₁₀ below 2.5 μm in diameter, because these particles can penetrate to the deep lung. Also, the chemical composition of this fine particle fraction is less variable, across geographic regions, than is the composition of the large particle fraction, and previous laboratory work has demonstrated toxic effects associated with specific components of the fine particle fraction; notably acidic sulfates and combustion-related carbonaceous aerosols. The present study analyzed the effects of two PM₁₀ components, ammonium bisulfate (ABS) and elemental carbon (C), which were selected because they represent important fractions of ambient PM₁₀ aerosols. The concentrations of each component were representative of estimated peak 4 hr concentrations, based on extrapolations from ambient air data, and the sizes of the particles used were chosen based upon reported sizes of fine inorganic aerosols in ambient air.⁶² Exposures were performed in the presence and absence of ozone (O₃; 0.2 ppm) because O₃ is often present in particle-contaminated atmospheres in California. Several biological endpoints were measured, each of which could be related to specific pulmonary diseases or disease processes. For example, changes in airway permeability, infiltration of the lung by inflammatory cells and mucus glycoprotein expression were measured to examine mechanisms related to lung inflammation and edema. Histopathological alterations in the lung, as indexed by post-exposure increases in cell replication, were measured to identify sites of lung injury related to repeated exposures. Macrophage function changes, which are related to host defenses (macrophage Fc receptor binding, phagocytic capacity and release of biocidal reactive oxygen species, e.g. superoxide anion) were measured because impairment of these functions can relate to increased risk of lung infection.

The mechanisms by which inhalation of ambient particles precipitate human fatalities are not clear, however some likely possibilities have been postulated. For example, inhaled particles might impair the integrity of the lung's epithelial barrier causing increased infiltration of cells, serum and proteins into the lung ultimately leading to inflammatory responses, pulmonary edema and death. Under the conditions of this study, significant permeability changes were observed 12 hr post-exposure, in the absence of evidence of lung infiltration by inflammatory cells. Previous studies of younger rats, but at higher particle concentrations had shown both permeability changes and PMN infiltration. Impairment of pulmonary host defenses by inhaled particles could permit

development of acute respiratory infections, or could cause acute inflammatory reactions with the release of excess amounts reactive oxygen species and cytotoxic chemicals in the lung. These releases could exacerbate existing chronic pulmonary diseases. In a previous study with young adult rats exposed to non-acidic ammonium sulfate at the same concentrations as used for ABS in this study⁶³, there was a tendency (which was not statistically significant) for macrophages from PM10 component-exposed rats to generate excess superoxide. We demonstrated in the present study that PM exposure increased free radical production immediately post-exposure, but that both respiratory burst and Fc receptor binding were depressed in macrophages sampled 12 hr post-exposure. These findings are consistent with those of Jakab et al.,⁶¹ that also indicated particle-induced impairment of host defenses. However, they assessed the ability of macrophages to kill ingested bacteria while we assessed mechanisms related to host defense functions.

Ruiz et al.⁶⁴ have reported that macrophages from children exposed to high environmental levels of airborne particles in Santiago, Chile show increased production of reactive oxygen species (ROS) compounds with concomitant reduction of circulating antioxidant compounds, indicative of oxidative stress. Oxidative stress following exposure to oxidant compounds in ambient air may be linked with the development of cardiopulmonary and cardiovascular diseases.⁶⁵ The present study examined only lung disease-related parameters, and did not explicitly examine possible links between PM exposures and the development of heart disease. This should be included in future studies.

Our earlier studies with ammonium sulfate (AMS)⁵⁷ and the results of our Phase 1 study with ABS + O₃ showed increased ROS production in macrophages from rats lavaged immediately after exposure. On the other hand, the ROS production of macrophages lavaged from rats 12 hr post-exposure to ABS + O₃ was depressed. These results suggest that the time course of macrophage responses may include an initial excitatory phase during which time oxidative stress can occur and that subsequent to this, the macrophages may be depressed in function. There is a need for additional studies to examine these mechanisms in greater detail because they could have important implications with respect to particle-associated impairment of host defenses.

An interesting question which can be raised is whether or not the aged animals used in this study were inherently more susceptible to the effects of inhaled particles? Other investigators^{66, 67} had previously demonstrated that aged rats are more susceptible to the effects of O₃ exposure. Vincent et al.⁶⁸ demonstrated that senescent animals had higher increases of

interleukin-6 (up to 10-fold higher) and N-acetyl-beta-D-glucosaminidase (NAGA; 2-fold higher) in lung lavage after ozone exposure than did young adult or juvenile rats, suggesting age-related modifications in inflammatory and oxidative stress responses. The susceptibility question was partially addressed in this study, although aged and young adult rats were not exposed to PM atmospheres at the same time. In a previously reported study from our laboratory, young adult (2 month old) rats were exposed to particle + O₃ atmospheres, albeit for 8 rather than 4 weeks.²⁷ Qualitatively, the patterns of injury in young adult rats from our prior study may be different from those in the aged rats in the present study. For example, the respiratory burst data suggests that young adult rats respond with lesser intensity to PM₁₀ exposures than do aged rats. This coupled with decreased anti-oxidant defenses in the senescent rat could render these animals more susceptible to oxidative stress and to PM-induced lung injury. Also, in young adult rats, lung injury included thickening of the alveolar walls, inflammatory changes, increased lung collagen content and increased permeability.⁶³ In the senescent rats we observed delayed permeability changes, no evidence of inflammatory cell infiltration and decreases in lung collagen content. Parameters relevant to infection susceptibility were affected in both young adult and old rats. Production of mucus glycoprotein was increased in young adult rats but not in old rats. Taken as a whole, these differences suggest that young adult rats are better able to mount defenses against particle-induced injury than are the senescent rats.

This study did not examine chronic effects, and it is an open question as to whether human populations with high ambient exposures to PM₁₀ might exhibit decreased lung compliance and possibly decreased pulmonary function as measured by forced expiratory maneuvers. While some epidemiological study findings might support this contention, the specific role played by PM₁₀ as compared to that played by oxidant gases such as O₃ is not clear. This is because acute O₃ exposure provokes many of the same effects as caused by the particles, and, in the environment, the same populations might be exposed to both high PM₁₀ and high O₃ concentrations (albeit not necessarily at the same time). In this study, however, at low concentrations, only atmospheres which contained both O₃ and acidic particles produced statistically significant biological responses. This, coupled with recent epidemiological findings that suggest a joint O₃ and PM effect, emphasizes that effects of pollutant mixtures can be important and that studies in which only single compounds are tested may understate the effects of particles on health.

The dose-response Phase 2 exposures indicated that while macrophage-related responses were suppressed in a dose-dependent manner, effects for some endpoints did not tend to increase in the rats exposed to higher concentrations of PM components, compared to those in rats exposed to the lower concentration of particles. This was evident in nearly all the non-macrophage endpoints. We examined the possible role of experimental bias as an explanation for this finding, however the exposures were well-controlled and simultaneous, the rats were randomized during endpoint evaluations and this was a blinded study. Experiment bias is therefore not likely to explain the observed attenuation. The results suggest that some injury mechanisms vary as a function of dose and alter the intensity or timing of toxic responses. Thus, more in-depth examinations of acute effects as a function of dose are needed. The endpoints showing attenuated response with increased dose were those requiring active metabolic interactions. It is possible that mild toxicity provoked by low concentration exposures induced repair or defense responses and that these responses were blocked or saturated during high dose exposures. Another possibility is that the time course of injury was altered or that some saturation of response mechanisms occurred. These possibilities should be further explored. Additional studies of intermediate concentrations that bracket those relevant to ambient exposures and a range of exposure/evaluation times could help elucidate injury mechanisms and their dose-response relationships.

Summary and Conclusions

Summary

The purpose of this study was to evaluate mechanism-related biological responses to particle-ozone mixture exposures that have been implicated as potentially causing human mortality and morbidity. This study examined the effects of PM exposures using aged (22 to 24 months), senescent rats as laboratory models of the sensitive, aging human population. Controlled (4 hr/day, 3 d/wk, 4 wk) nose-only exposures were performed using two components which are prevalent in the fine particle fraction of PM, carbon (C; 50 $\mu\text{g}/\text{m}^3$) and ammonium bisulfate (ABS; 70 $\mu\text{g}/\text{m}^3$). The particles were about 0.3 μm mass median aerodynamic diameter. Particle exposures were performed in the presence and absence of ozone (O_3 ; 0.2 ppm) because O_3 is an important constituent of urban aerosols in California and high concentrations of O_3 often occur in conjunction with high concentrations of particles. Control atmospheres were purified air and O_3 alone. The

project was performed in two phases. Phase 1 examined the toxicity of PM components alone and in mixtures, at the concentrations stated above. Phase 2 evaluated mechanisms of injury as a function of particle concentrations. Two concentrations of C + ABS + O₃ were used in Phase 2 (Low = 50 µg/m³ C, 70 µg/m³ ABS, 0.2 ppm O₃ and High = 100 µg/m³ C, 140 µg/m³ ABS, 0.2 ppm O₃).

Phase 1: Effects of Specific Components

Particle-containing atmospheres produced lung injury, measured as the labeling of DNA in lung epithelial and interstitial cells undergoing replication at the end of the 4-week exposure period. However, group mean value differences were significant only in the group of rats exposed to the atmosphere containing the combination of ABS, O₃ and C, compared to the purified air controls. Although the epithelial cells were most likely initially impacted by inhaled particles, the adjacent interstitial cells were more intensively injured, due to secondary processes.

Macrophages from rats exposed to PM10 components exhibited slightly, but not significantly, depressed ability to attack antigenic material (SRBC) via Fc receptor-mediated processes. The production of superoxide during respiratory burst activity following stimulation with opsonized zymosan was increased by exposures to the particle-containing atmospheres, and superoxide production following exposures to the ABS + C + O₃ mixture was significantly greater than that observed for O₃ alone. Phagocytic activity was slightly elevated after particle exposures and slightly depressed after ozone exposure. However, combined particle plus O₃ exposures resulted in significantly ($p \leq 0.05$) increased phagocytic activity.

None of the Phase 1 exposures significantly increased permeability (measured as bronchoalveolar lavage (BAL) fluid concentrations of either total protein or albumin), in the presence or absence of O₃. The data indicated a trend towards higher levels of proteins and mucus glycoprotein in BAL following exposures to particle plus O₃ mixtures. Phase 1 examined BAL collected immediately post-exposure, as had been done previously with young adult rats. Phase 2 studies examined permeability 12 hr post-exposure, to determine if in senescent rats the permeability occurred at a later time.

Phase 2: Effects of PM Concentration

The Phase 2 was designed after evaluation of the Phase 1 findings and specifically addressed relationships between exposure concentrations and biological responses. The lavage-

related endpoints were conducted at 12 hr post-exposure, rather than immediately post-exposure, as had been done in Phase 1, to improve the sensitivity of some assays.

Lung injury was assessed by determining rates of cell replication in epithelial and interstitial tissue. Ozone increased cell replication rates 20 to 40% in interstitial and epithelial cells, but cell replication rates in rats exposed to particle plus ozone mixtures were increased by between 250 to 340%, relative to purified air. The data confirmed the Phase 1 finding that more cell replication was induced by particle mixture exposures in the interstitial region, as compared to the epithelial region, relative to the baseline replication rate (*i.e.* that seen after purified air exposures). The low concentration mixture induced the most intense injury (significant vs. purified air), but was not significantly different from that induced by the high concentration mixture.

Macrophages from rats exposed to both the high and low concentrations of the PM10 component + O₃ mixtures exhibited significantly depressed ability to attack antigenic material (sheep red blood cells) via Fc receptor-mediated processes. The production of superoxide during respiratory burst activity following stimulation with opsonized zymosan was depressed by exposures to both concentrations of the particle-containing atmospheres relative to either purified air or O₃. Comparisons made between macrophage function endpoints in rats euthanized immediately post-exposure and rats euthanized 12 hr post-exposure suggest that macrophages were activated immediately after particle exposure, but that they become functionally depressed within 12 hr post-exposure.

Permeability was slightly increased by ozone and significantly increased by exposure to the low concentration particle + O₃ mixture when lung epithelial permeability was measured 12 hr post-exposure. There was no evidence of inflammatory cell infiltration, although there was a general tendency for O₃-containing atmospheres to slightly, but not significantly, increase PMN concentrations in BAL. The effects of high and low concentration PM exposures on permeability, mucus glycoprotein release and most other endpoints were not significantly different from each other, although there were significant differences vs. purified air.

Conclusions

Exposure to mixtures of fine elemental carbon and ammonium bisulfate particles and ozone produced deleterious pulmonary effects in these aged rats following repeated exposures. Exposures to submicron PM components injured epithelial and interstitial cells of the lung parenchyma, and

altered macrophage functions related to defenses against respiratory infections. The magnitudes of the observed changes were small, but it should be understood that despite their age, these 22 to 24 months old animals were free of prior respiratory infections and were in relatively good health. The addition of O₃ to the particle mixture significantly increased the magnitudes of several of the measured biological responses, and, in fact, only those atmospheres that contained both particles and O₃ evoked statistically significant differences.

This study also demonstrated that, for most endpoints, effects after low (50 µg/m³ C and 70 µg/m³ ABS) concentration PM exposure were not lower than those after high (100 µg/m³ C and 140 µg/m³ ABS) concentration PM exposure. This finding could suggest saturation of response mechanisms, or it may be that effects seen at low concentration represent either defensive or injury-repair responses and that these responses are blocked or suppressed after higher concentration exposures. Alternatively, response latency may have been altered as a function of dose. If so, greater effects at high concentrations might have been noted at endpoint evaluation times other than those used in this study.

This study provides some important new insights into the mechanisms by which inhaled particles might cause the significant health outcomes associated in epidemiological studies of ambient PM exposures. Overall, this study supports the concept that PM exposure can increase lung permeability and can thus potentially contribute to edema and the transfer of materials across the lung's epithelial barrier. Also, this study demonstrated that, in conjunction with O₃, PM exposure causes statistically significant injury to lung tissues and can increase potential risks of respiratory system infections by interfering with macrophage-dependent host defenses. These are plausible biological mechanisms by which PM exposure might be related to human morbidity and perhaps mortality.

Recommendations

1. This and prior laboratory studies have shown that certain mixtures containing particles and ozone are more toxic than either particles alone or ozone alone. Recent epidemiological studies have shown that in some cases there are similar interactions between ozone and particles that provoke deleterious effects in humans. Studies are needed to delineate the mechanisms by

which oxidant plus particle interactions affect health. Although ozone is an important oxidant, other ambient contaminants such as nitrogen oxides, peroxides and transition metal have oxidant properties and can produce free radicals in the lung. These should be evaluated in future studies.

2. Other ambient compounds, notably sulfur and nitrogen oxides and carbon monoxide, have been identified as possible "confounders" in epidemiological studies of particle-induced morbidity and mortality. Mechanisms by which these compounds can mediate particle-induced health effects could be examined in laboratory studies using suitable animal models. Ethical considerations might preclude human studies of this nature with sensitive populations at this time.
3. The influence of particle size on toxicity of PM components is an important variable that has not been examined using realistic particles in suitable inhalation studies. Studies of the dependence of particle toxicity on both size and concentration are needed for a variety of compounds that contribute to ambient PM.
4. Mechanisms relevant to the attenuation of effects at elevated concentrations need to be examined to determine how toxic responses impair repair and defense processes in the lung.
5. Additional studies that include both pulmonary and cardiological endpoints are needed because, in epidemiological studies, PM exposures increase the risk of heart-associated death, and because of the close integration of respiratory and cardiac functions.
6. Although the current research is limited to acute responses, chronic effects of PM exposure and the time-course of PM-induced injuries should be evaluated.
7. Studies should be performed using genetically altered animal models that closely simulate characteristics of susceptible humans to elucidate mechanisms of injury at the molecular level, *in vivo*.

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**EFFECTS OF REPEATED EXPOSURES OF GERIATRIC RATS
TO OZONE AND PARTICLE-CONTAINING ATMOSPHERES:
AN ANALYSIS OF BRONCHOALVEOLAR LAVAGE
AND PLASMA PROTEINS**

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Inhalation of ozone (O₃) and airborne particles less than 10 µm (PM-10) in mass median aerodynamic diameter (MMAD) is associated with adverse health effects in sensitive human populations, and pulmonary injury in laboratory animals. In order to simulate environmental exposures of sensitive individuals, such as the elderly, geriatric rats were exposed to O₃ and particulate mixtures. This pilot study determined whether the pulmonary effects of higher O₃ concentrations reported in earlier studies are also seen after repeated exposures to lower O₃ concentration used in this study, whether the O₃ effects are modified by PM-10 components, and whether plasma biomarkers can be developed as noninvasive tests of pulmonary injury. Male Fischer 344 rats, 22–24 mo old, were exposed 4 h/day, 3 days/wk for 4 wk to (a) purified air, (b) O₃ (0.2 ppm), (c) low-level mixture of carbon (C, 50 µg/m³) plus ammonium bisulfate (ABS, 70 µg/m³) plus O₃, and (d) high-level mixture of C (100 µg/m³) plus ABS (140 µg/m³) plus O₃. Twenty-four hours after the last exposure, groups of rats were prepared for measurement of protein and albumin concentrations in bronchoalveolar lavage (BAL) as markers of airways permeability, and blood was drawn for analysis of plasma immunoreactive prolyl 4-hydroxylase and fibronectin. Exposures to O₃ and O₃/particle mixtures did not produce a consistent, significant change in the BAL markers of permeability. Nonsignificant changes represented individual animal variations. On the other hand, a significant increase in plasma fibronectin was observed in the group exposed to O₃, but not in the rats exposed to O₃/particle combinations. Such an effect was not observed for plasma immunoreactive prolyl 4-hydroxylase. The inability of low-level O₃ and O₃/particle combinations to produce intrapulmonary effects suggests potential utility of plasma biomarkers for the evaluation of pulmonary toxicity. These results also suggest modification of O₃ effects upon its combination with PM-10.

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Ozone (O_3), the principal oxidant in photochemical smog, induces lung injury and alters lung function in both animals and humans (U.S. EPA, 1986; Lippmann, 1989; Devlin et al., 1991). While a brief ozone exposure damages respiratory cells, initiates neutrophilic inflammation (Bhalla & Young, 1992), and increases collagen synthesis rates (Last et al., 1984a; Hesterberg & Last, 1981), a prolonged exposure is regarded as a contributing factor to the incidence and severity of respiratory disease (Last et al., 1984b). Chronic exposure to ambient levels of O_3 is of considerable interest to the general public who continuously breathe polluted atmospheres and also to workers who are occupationally exposed to this pneumotoxicant. Particulate matter in the air constitutes another important component of smog-associated adverse health effects. Inhalation of airborne particles of less than 10 μm in aerodynamic diameter (PM-10) is associated with increased hospital admissions and emergency room visits for respiratory disorders (Martin, 1964; Greenburg et al., 1967; Samet et al., 1981; Knight et al., 1989), increased numbers of asthma attacks, and increased usage of asthma medication (Schenker, 1993). In epidemiologic studies, PM-10 inhalation was associated with reduced pulmonary function (Stern et al., 1989; Pope & Kanner, 1993) and increased mortality (Schwartz, 1993; Dockery et al., 1993). People above 60 yr of age and those with preexisting cardiovascular or chronic pulmonary disorders are the major victims of PM-10 toxicity. This group of people shows a significant increase in total annual mortality rates when exposed to ambient fine particles and sulfates (Ozkaynak & Spengler, 1985; Ozkaynak & Thurston, 1987). To test the sensitivity of the aged populations to O_3 - and PM-10-containing atmospheres, 22-mo-old geriatric rats were exposed to O_3 and mixtures of carbon, ammonium bisulfate, and O_3 repeatedly for 4 wk. This study represents an attempt to find specific biological markers of pulmonary injury, especially plasma biochemical markers, which can be used as frequent and noninvasive tests of O_3 - or PM-10-related injury.

Collagen, comprising 10–20% of the dry weight of the lung (Crystal, 1974), is an integral part of interstitial lung connective tissue. In the lungs of normal adults, the amount and types of collagen synthesized are regulated throughout development and are only altered in lung disorders. A method employing measurement of plasma/serum prolyl hydroxylase was employed to detect possible increases in stimulation of collagen synthesis in the lung following PM-10 or O_3 inhalation. Since plasma fibronectin has been shown to be a good indicator of pathological states with injury of the reticuloendothelial system (Saba & Jaffe, 1980; Saba et al., 1986), this study also investigated the usefulness of plasma fibronectin in the assessment of PM-10- and O_3 -induced lung injury. In order to investigate a relationship between plasma and pulmonary changes following pollutant exposures, broncho-

alveolar lavage (BAL) fluid was analyzed for total protein and albumin levels. Previous studies from this laboratory (Bhalla & Young, 1992) have shown that acute exposures to O₃ concentrations higher than those used in this study disrupt the air-blood barrier and result in increased accumulation of total protein and albumin in air spaces. The present study was aimed at determining if similar changes are produced by repeated exposures to lower pollutant concentrations. While the total protein could be a combined measure of secretory proteins released by damaged and stimulated cells and transport from blood (Su et al., 1991), albumin concentration presumably represents transport from blood to airways. Therefore, both of these measures were included in this study to improve the sensitivity for detecting pollutant effects.

METHODS

Animal Exposures and Atmospheres

Animals Barrier-reared male Fischer 344 NNia rats, 22–24 mo old and weighing about 400 g, were purchased from the NIA colony at Harlan Sprague Dawley (Indianapolis, IN). Animals were shipped by the supplier in filtered containers to minimize exposure to particulate pollutants during the transit. The animal cages were held in a laminar-flow, air-barrier caging system prior to their use in the study.

Experimental Design Rats were randomly assigned to exposure groups on arrival at our laboratory. Rats were exposed, nose-only, to 1 of 4 exposure atmospheres: (a) purified air, (b) O₃ (0.2 ppm), (c) low-level mixture of carbon (C, 50 µg/m³) plus ammonium bisulfate (ABS, 70 µg/m³) plus O₃, and (d) high-level mixture of C (100 µg/m³) plus ABS (140 µg/m³) plus O₃. All exposures were 4 h/day, 3 consecutive days/wk for 4 wk. Exposures were conducted simultaneously in four matched exposure chambers so that animal handling and effects of diurnal or seasonal influence in response or behavior were uniform over the study. Nose-only exposures were required in order to prevent artifacts due to airborne dander, ammonia, and dried excreta. Between exposures, rats lived in purified-air barrier housing and they were given clean water and dry laboratory feed ad libitum. Animals were housed in wire cages over beds of rock salts, which dried feces and urine and suppressed dust and ammonia production. Animals were handled by clean-clothed, masked, gloved personnel to prevent the spread of infections to the rats. Exposure tubes were inspected daily and thoroughly machine-washed in hot soapy water daily. Animal cages were washed and sterilized, and quarters were cleaned twice weekly.

Pollution Generation Ozone was generated by passing medical grade oxygen through two corona-discharge ozonizers (Sander ozonizer, type III, Osterberg, Federal Republic of Germany). The dry carbon par-

ticles (Monarch 120 carbon black particles; Cabot Corp., Boston) were suspended in an aqueous solution of ABS, agitated ultrasonically, and nebulized with compressed air. The aerosol was dried by rapid dilution, discharged to Boltzmann equilibrium using ^{85}Kr sources, and introduced into the chamber air supply. The nebulization process produced particles of carbon or ABS with $0.4\ \mu\text{m}$ mass median aerodynamic diameter (MMAD) and a geometric standard deviation (measured by cascade impactor) of about 2.0. This mean is in the range of sizes in which these are formed in the air.

Pollutant Characterization Aerosol particles were collected on preweighed and equilibrated (50% relative humidity) Pallflex T60A20 Teflon-coated glass fiber filters (PALL Corp., Cincinnati, OH). The filters were weighed after collection to determine the total collected mass. Pallflex filters were extracted with dilute carbonate/bicarbonate buffer and the extract was analyzed for SO_4^{2-} by ion chromatography.

Samples for carbon analysis were collected on acid-treated quartz fiber filters (Microquartz, Gelman, Ann Arbor, MI). Carbon was determined by combustion in oxygen in a flow-through furnace and measurement of the evolved CO_2 using a nondispersive infrared absorption analyzer (Dasibi model 3003, modified with a CO_2 absorption cell).

Lavage Fluid Analysis

Bronchoalveolar Lavage The lavage procedure was similar to that described in the previous studies (Kleinman et al., 1995). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital, 50 mg/kg, 12–16 h after the end of the last exposure. The abdominal aorta was severed and a polyethylene catheter was placed and tied in the trachea. An incision was made in the diaphragm to allow lung expansion during the lavage. Lungs were lavaged by introduction of 7 ml HEPES-buffered Hanks balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} through the tracheal catheter followed by withdrawal of the fluid. The process of introduction and withdrawal of lavage fluid was repeated 3 times, and at least 5 ml lavage fluid was recovered and transferred to a 15-ml polypropylene centrifuge tube. The lavage from each animal was centrifuged at $300 \times g$ for 10 min. The supernatant from the lavage, lacking cells, was analyzed for total protein and serum albumin.

Protein Measurement in Bronchoalveolar Lavage A protein assay kit utilizing bicinchoninic acid (BCA) as the working reagent (Pierce BCA protein assay reagent, Pierce Chemical Co., Rockford, IL) was used. A set of appropriately diluted samples and protein standards prepared by diluting a stock solution of bovine serum albumin (BSA) were processed following Pierce's published microtiter plate protocol. Sample absorbance was read at 560 nm with a microtiter plate reader (Tecan/SLT Lab Instruments, Hillsborough, NC). The standards read for

each plate were used to prepare a standard curve by plotting the net absorbance at 560 nm versus protein concentration. The protein concentration for each unknown sample was calculated by linear regression using the standard curve.

Albumin in Bronchoalveolar Lavage Enzyme-linked immunosorbent assay (ELISA) procedures described by Schwerer et al. (1987) and Macy et al. (1988) were used as a guide for establishing an albumin assay. Polystyrene nonflexible 96-well microtiter plates (Costar, Van Nuys, CA) were coated with goat anti-rat antibody to albumin (Organon Teknika, Durham, NC) in carbonate buffer, pH 9.6. The plates were covered, refrigerated overnight, and then washed with carbonate buffer to remove excess material or antibody. Nonspecific binding was blocked by the addition of gelatin in carbonate buffer. The plates were placed in a humid chamber at room temperature for 1 or 2 h and then washed 2 times with phosphate-buffered saline (PBS)-Tween 20-gelatin. Serial dilutions of a standard rat albumin (Sigma Chemical Co.) and rat lavage fluid in PBS-Tween 20-gelatin solution were added to each well. After an additional incubation and washing, peroxidase-conjugated rabbit anti-rat albumin antibody was added to each well. The plates were incubated in a humid chamber at room temperature for 1 h, and color was developed by the addition of 100 μ l per well of citrate-phosphate buffer (pH 5.0) containing 1 mg/ml O-phenylenediamine dihydrochloride (OPD) and 1 μ l/2 ml of 30% H₂O₂. The reaction was stopped by the addition of 2 N H₂SO₄. The plates were read at 492 nm in a Tecan/SIT Labinstruments microplate reader.

Plasma Analysis

Sample Collection Blood was drawn by cardiac puncture and collected in tubes containing potassium-ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. Plasma was prepared by centrifuging the blood samples at 1500 \times g for 10 min at 5°C. The plasma samples were divided into 1-ml aliquots and stored at -20°C until they were assayed for plasma immunoreactive prolyl 4-hydroxylase (IRPH) and plasma fibronectin.

Assay for Plasma Immunoreactive Prolyl 4-Hydroxylase The plasma concentration of IRPH was determined by an enzyme immunoassay (EIA) using a rat prolyl 4-hydroxylase kit (Fuji Chemical Industries, Ltd., Takaoka, Japan) (Yoshida et al., 1986). The assay was based on a one-step sandwich method employing specific monoclonal antibody against rat prolyl 4-hydroxylase β -subunit. The assays were carried out in duplicate. Plasma samples and standards (solutions of known concentration of pure rat IRPH protein were used to make a standard curve), 20 μ l, were incubated with 300 μ l peroxidase-labeled anti-rat IRPH monoclonal antibody solution and polystyrene beads (6.5 mm in

diameter) coated with an anti-rat IRPH at 25°C for 60 min. After incubation, the unbound peroxidase-labeled anti-rat IRPH antibody was removed by washing the polystyrene beads with saline and then transferring the beads into new test tubes. Then 300 μ l of coloring solution containing O-phenylenediamine (OPD) and 0.2% H₂O₂ was added to the test tube. Incubation for 30 min at 25°C was carried out and the enzyme reaction was stopped by adding 2 ml of 1.2 N H₂SO₄. The absorbance of the solution in the test tube was measured in a spectrophotometer at 492 nm. The activity of peroxidase was proportional to the amount of IRPH protein, so that rat IRPH concentration in plasma specimens could be determined from the standard curve. The plasma IRPH protein was expressed as micrograms per milliliter of plasma.

Electroimmunoassay for Plasma Fibronectin Plasma fibronectin levels were measured by electroimmunoassay using Laurell rocket immunoelectrophoresis (Blumenstock et al., 1977). An antiserum to rat fibronectin was mixed with a 1% agarose gel to yield a final antiserum concentration of about 0.6%. The antiserum to rat fibronectin was kindly provided by Dr. F. Blumenstock, of Albany Medical College of Union University, New York. For assay, 3-mm wells were cut in the agarose-coated 5 \times 10-in glass plates at intervals of 1 cm, and 10 μ l rat plasma was added to each well. Pure rat plasma fibronectin (Chemicon International Inc., Temecula, CA), 1 mg/ml, was used to standardize the assay. The samples were electrophoresed toward the anode at a voltage of 7 V/cm and 15°C for 20 h. The plates were washed and stained. Rocket heights were recorded in millimeters, and absolute concentration was determined by drawing a standard curve relating rocket height to varying concentrations of known rat plasma fibronectin standard. The plasma fibronectin concentration was expressed as micrograms per milliliter of plasma.

Statistics The data were collected from a total of 5 rats per group for plasma IRPH and fibronectin levels and from 10 rats per group for BAL protein and albumin concentrations, except for albumin in the high-concentration mixture group, which had a sample size of 9 instead of 10. The differences in control and experimental groups exposed to O₃ and O₃/PM-10 combinations were analyzed by one-way analysis of variance. The criterion for statistical significance was set at $p \leq .05$. Group mean values were further analyzed by the Tukey multiple comparison test.

RESULTS

Permeability measurements were performed to determine the effects of O₃ and selected combinations of PM-10-containing atmospheres on the airway epithelia of rats. The barrier functions of the airway epithe-

lium and its integrity following exposure of geriatric rats to test atmospheres were assessed by measuring levels of total protein and albumin in BAL. An increase in the levels of BAL proteins, especially albumin, is a reflection of epithelial barrier disruption. The results of this study did not reveal any significant change in BAL concentrations of either total protein or albumin following exposure to PM-10 components in the presence or absence of O₃ (Table 1) for any of the groups tested.

Plasma IRPH levels from groups of rats were compared for pollutant effects. Plasma IRPH concentrations, measured in all of the plasma samples collected from experimental and control animals, are shown in Table 1. The IRPH concentrations in samples recovered from animals exposed to O₃ and O₃/PM-10 combinations were not significantly different from the IRPH level of 0.57 µg/ml in air-exposed controls.

The plasma fibronectin levels in all of the groups are shown in Figure 1. The mean plasma fibronectin concentration in air-exposed rats was 770 µg/ml. The plasma fibronectin concentration of 930 µg/ml in the group exposed to 0.2 ppm O₃ was significantly greater than the corresponding levels in the samples obtained from the air-exposed controls. However, a similar increase in plasma fibronectin levels was not observed in the group exposed to the high-level O₃/PM-10 combination. The fibronectin level in the group exposed to the lower O₃/PM-10 combination was significantly lower than that in the control.

DISCUSSION

The stimulation of collagen synthesis in the lung due to air pollutant exposures can be measured by histological examination of lung biopsy, biochemical assays of homogenized lung tissue samples, or

TABLE 1. Plasma immunoreactive prolyl hydroxylase (IRPH) and bronchoalveolar lavage (BAL) protein and albumin concentrations in rats exposed to air, ozone, and ozone/particle combinations

Exposure	Biological markers		
	Plasma IRPH (µg/ml)	BAL protein (µg/ml)	BAL albumin (µg/ml)
Purified air	0.57 ± 0.04	84 ± 6	44 ± 9
Ozone (0.2 ppm) ^a	0.52 ± 0.06	97 ± 8	58 ± 12
Ozone + PM-10 ^b	0.58 ± 0.03	91 ± 8	46 ± 8
Ozone + PM-10 ^c	0.61 ± 0.04	84 ± 4	36 ± 7

Note. Each value represents the mean ± SE.

^aOzone: 0.2 ppm.

^bLow-level mixture: carbon (50 µg/m³) + ammonium bisulfate (70 µg/m³) + O₃ (0.2 ppm).

^cHigh-level mixture: carbon (100 µg/m³) + ammonium bisulfate (140 µg/m³) + O₃ (0.2 ppm).

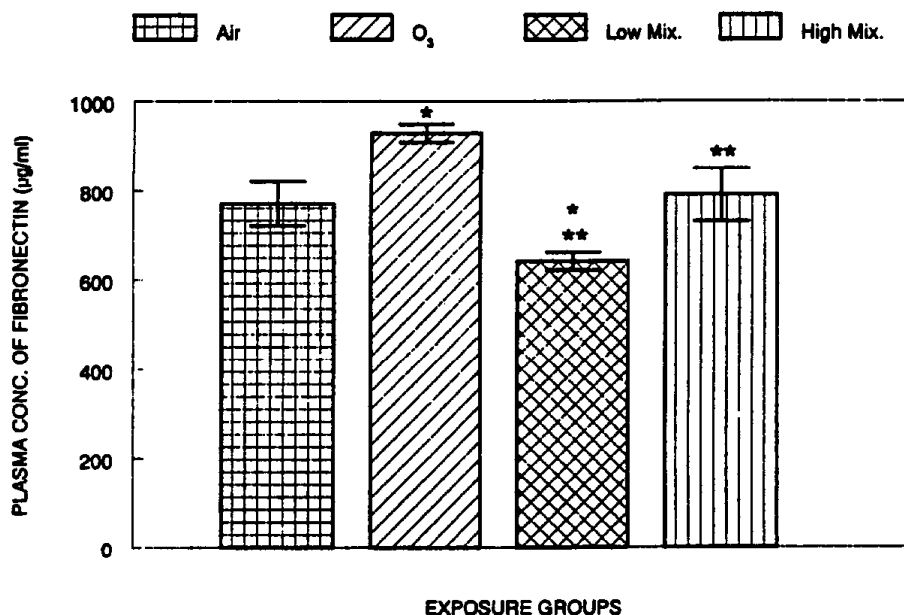


FIGURE 1. Plasma fibronectin in geriatric rats after repeated exposure to purified air, O₃ (0.2 ppm), a low-level mixture of carbon (C, 50 µg/m³) plus ammonium bisulfate (ABS, 70 µg/m³) plus O₃, and a high-level mixture of C (100 µg/m³) plus ABS (140 µg/m³) plus O₃. Means ± SE are shown. Single asterisk indicates a significant change ($p < .05$) in comparison to the control, and double asterisk indicates a significant decrease in comparison to O₃ group.

analysis of plasma/serum levels of prolyl 4-hydroxylase activity. Measurement of prolyl 4-hydroxylase activity in lung specimens has indicated increases in collagen formation in rats and guinea pigs exposed to 0.4 ppm and higher O₃ concentrations (Hussain et al., 1976; Kodavanti et al., 1995). Prolyl 4-hydroxylase protein exists in animal and human plasma as monomers and dimers, which are either nonactive or less active than the tetramer in the cytoplasm (Bolarin, 1988). It has been reported that an inhibitor to the enzyme exists in the serum (Chichester & Fuller, 1980). Thus, serum or plasma assays of enzymatic activity have been considered less valuable in the diagnosis or monitoring of fibrotic changes in tissues. Therefore, this study utilized an analysis of IRPH instead of a measurement of the enzyme activity. The recent development of monoclonal antibodies to human and rat prolyl 4-hydroxylase β -subunit (Yoshida et al., 1986) permitted measurement of the serum or plasma IRPH using commercially available enzyme immunoassay kits. Studies utilizing this assay have reported increased serum or plasma levels of IRPH in patients with chronic hepatitis, liver cirrhosis, alcoholic liver damage, and other liver diseases, suggesting the usefulness of IRPH as a potential and early indicator of fibrotic

changes (Yoshida et al., 1986; Murawaki et al., 1991). However, our study did not reveal a change in plasma IRPH concentrations following repeated exposures of rats to O₃ alone or to PM-10 and O₃ in combination. However, these data indicate that plasma IRPH protein, unlike plasma prolyl hydroxylase activity, can be easily measured.

Fibronectin is a large, dimeric glycoprotein (about 440,000 Da) found in plasma, other biological fluids, loose connective tissues, and some basal lamina. It is synthesized by a variety of cells including lung fibroblasts, endothelial cells, macrophages, and type II alveolar epithelial cells. Fibronectin has numerous biological functions relating to specific binding domains for cells and for extracellular macromolecules including collagens. Plasma fibronectin has been shown to be a good indicator of pathological conditions associated with injury of the reticuloendothelial system (Saba & Jaffe, 1980), radiation-induced lung injury (Lafuma et al., 1987), and in patients with bronchopulmonary carcinoma (Raffi et al., 1983). A number of recent studies have also shown that a single or repeated daily exposures to O₃ result in an increase in BAL fluid fibronectin concentration and concomitant decrements in lung function in humans and animals (Kodavanti et al., 1995; Bouthillier et al., 1996; Devlin et al., 1991). Our results indicate that a significant increase in plasma fibronectin also occurs following O₃ exposure. Previous studies have demonstrated that BAL and plasma fibronectin are immunologically similar and antigenically indistinguishable (Villiger et al., 1981). This raises the possibility that lung components contribute to the plasma fibronectin. In general, regardless of the causative agent, the lung responds to insults in a stereotypical manner. Pulmonary injury caused by such diverse agents as ozone, cigarette smoke, radiation, acid, drugs, or multiorgan diseases results in a generalized activation of counterreactive mechanisms, which include release of inflammatory mediators such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and fibronectin. Fibronectin synthesis is increased in macrophages and polymorphonuclear leukocytes isolated from inflammatory foci. Fibronectin is involved in many complex functions, some of which include the enhancement of the binding of a lymphokine to macrophage and the expression of Fc receptors on macrophages. It also increases adherence and chemotaxis of phagocytes and helps maintain the oxidative bactericidal capacity of macrophages.

Although the results of this study demonstrate that exposures to O₃ concentrations that approach ambient levels do not produce detectable changes in the lung, as indicated by the lack of O₃ effect on BAL protein and albumin levels, the 21% increase in plasma levels of fibronectin represents an extrapulmonary pathologic response to low-level O₃ exposure. Though the origin of the increased plasma fibronectin is not known, lung fibroblasts and macrophages are among

the potential sources contributing to increased plasma fibronectin following O_3 exposure. Fibronectin is a major secretory product of lung fibroblasts (Yamada & Olden, 1978), which represent 35–40% of the parenchymal cell population of normal lung (Crapo et al., 1980). Fibronectin is also produced by alveolar macrophages, a cell type representing 90% of the inflammatory and immune cells of the lower respiratory tract. The studies demonstrating fibronectin synthesis by liver cells in response to inflammation (Owens & Cimino, 1982) suggest that increased hepatic synthesis of fibronectin in response to O_3 -induced inflammatory reactions could also contribute to higher plasma fibronectin levels. The ozone-induced lung injury leading to changes in pulmonary endothelial cells constitutes another potential source for the release of cellular fibronectin. These cells are known to increase plasma total fibronectin concentration (Saba & Jaffe, 1980). Although the low levels of O_3 exposure utilized in our study are not likely to produce gross endothelial changes in the lung, these exposures were effective in inducing plasma fibronectin. These results complement other studies suggesting an involvement of fibronectin in the development of acute lung injury and inflammation (Devlin et al., 1991). In these studies, Devlin et al. (1991) showed an elevation of BAL fluid total protein and fibronectin in humans exposed to O_3 .

In conclusion, the results of the present study demonstrate increased expression of fibronectin following repeated exposure of geriatric rats to O_3 , but not after exposure to O_3 /PM-10 combinations. These fibronectin effects appeared in the absence of altered plasma IRPH levels or BAL protein and albumin concentrations, which normally increase following exposure to higher O_3 concentrations. The measurement of total plasma fibronectin levels offers a potentially sensitive tool for detecting O_3 effects. The pilot data presented here also form the basis for further confirmatory studies leading to development of noninvasive biochemical markers to monitor minimal changes in the lungs following pollutant exposures.

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INTRA- AND EXTRA-PULMONARY EFFECTS OF REPEATED EXPOSURES OF GERIATRIC RATS TO OZONE AND PM-10 ATMOSPHERES. DM Bolarin¹, DK Bhalla² and MT Kleinman³. Depts. of Pharm. Sci.¹, Occup. & Environ. Health Sci.², Wayne State Univ., Detroit, MI and Comm. & Environ. Med.³, Univ. of California, Irvine, CA.

Inhalation of ozone (O₃) and airborne particles less than 10 µm diameter (PM-10) is associated with adverse health effects in humans, especially sensitive populations. To simulate environmental exposures of sensitive individuals, such as the elderly, geriatric rats were exposed to O₃ and PM-10 mixtures. This study was aimed at determining: 1) the pulmonary effects of repeated exposures of rats to near-ambient O₃ concentrations, 2) modification of O₃ effects by PM-10 components and 3) potential plasma biomarkers of pulmonary injury. Male F 344 rats, 22-24 months old, were exposed 4 hrs/d, 3 d/wk for 4 weeks to (a) air, (b) O₃ (0.2 ppm), (c) low level mixture, i.e., Carbon (C; 50 mg/m³) + Ammonium bisulfate (ABS; 70 mg/m³) + O₃ and (d) high level mixture, i.e., C (100 mg/m³) + ABS (140 mg/m³) + O₃. Bronchoalveolar lavage (BAL), recovered at 24 hrs after the end of last exposure, was analyzed for protein and albumin concentrations as markers of airway permeability, and blood was analyzed for plasma immunoreactive prolyl hydroxylase (IRPH) and fibronectin. Exposures to O₃ and O₃/PM-10 mixtures did not produce a significant change in the BAL markers of permeability or plasma IRPH. However, O₃, but not O₃/PM-10 mixtures, produced a significant increase in plasma fibronectin levels. Limited extra-pulmonary effects suggest potential utility of plasma biomarkers for evaluation of pulmonary toxicity. The results also suggest modification of O₃ effects upon its combination with PM-10. Supported by California ARB 93-318 and NIEHS ES03521.

PRESENTER: Michael T. Kleinman

TITLE: Toxicity of Constituents of PM10 Inhaled by Aged Rats

AUTHORS: Kleinman, MT, Mautz, WJ, Phalen, RF and Bhalla, DK

ABSTRACT

The EPA has estimated that exposures to particles smaller than 10 μm in mass median aerodynamic diameter (MMAD) (PM10) may contribute to approximately 60,000 deaths per year, based upon epidemiological associations. The biological mechanisms for effects of components of PM10 on human health are not firmly established. There is evidence that acidic and combustion-generated components, which are found in the fine particle fraction ($\leq 2.5 \mu\text{m}$ diameter) are among those which are the most toxic.

This study examined the toxicology of PM10 components using laboratory-generated aerosols containing acidic ammonium bisulfate (ABS), and resuspended carbon black (C), a surrogate for combustion-generated carbonaceous aerosols. Since the human population most affected by PM10 appears to be elderly, this study used geriatric rats (about 24 months old) as a model. *In vivo* subchronic 4 week nose-only exposures (4 hr/d, 3 d/wk) of rats ($n=10$ per endpoint per atmosphere) were performed with atmospheres containing either C or ABS at concentrations of 60 and 70 $\mu\text{g}/\text{m}^3$, respectively, or to a mixture of C + ABS. Ozone (O_3), might be an important co-pollutant with PM10, therefore mixtures of C + O_3 and C + ABS + O_3 were also tested. Control groups were exposed to purified air or to O_3 alone. The particle size was 0.5 μm mass median aerodynamic diameter, for both C and ABS aerosols. The following hypotheses were examined: (1) The mixture of carbon and acidic sulfate particles will have greater deleterious effects than will the carbon particles alone; and (2) The effects of the acidic sulfate and carbon particle mixture combined with 0.15 ppm O_3 will be more deleterious than the additive effects of the particle mixture and the O_3 portions of the mixture.

Biological endpoints which are related to the etiology or presentation of lung diseases were examined. These included permeability, inflammatory responses, macrophage functions, collagen synthesis, mucus production, and lung morphometry. Lung permeability was not significantly changed following exposures to any of the pollutant atmospheres, nor was there significant evidence of infiltration of alveolar spaces by inflammatory cells. Exposures to particle atmospheres, in the absence of O_3 , did not significantly affect macrophage functions, however macrophages from rats exposed to either C + O_3 , or C + ABS + O_3 showed functional changes that were significantly altered in comparison to those from rats exposed to O_3 alone. The O_3 -containing mixtures induced significant changes in collagen concentrations in lung tissue and resulted in significant increases in lung cell turnover rates; both changes are suggestive of irritant effects on the lung. Changes in both of these parameters were greatest following exposures to the mixture which contained ABS.

This study demonstrated significant biological effects in geriatric rats due to exposure to atmospheres containing components of the fine-particle fraction of PM10, i.e. carbon and ammonium bisulfate particles. Ozone (0.15 ppm) exacerbated the effects of this mixture. The results of the study supported both hypotheses. (Supported by the California Air Resources Board)

S3.3

CELLULAR AND IMMUNOLOGIC INJURY WITH PM10 INHALATION.

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PM10, or airborne particles less than 10 μm in mass median aerodynamic diameter (MMAD), are associated with adverse effects on human health including chronic lung diseases and mortality, but the mechanisms by which these particles cause or aggravate diseases are not specifically known. PM10 represents a complex mixture, both in terms of size and chemical composition, of aqueous-media soluble and insoluble particles. Furthermore, the ambient aerosol composition varies markedly in different locations and at different times in the same location. To test the effects of PM10 on pulmonary defenses in relation to specific cell targets, barrier-reared Sprague-Dawley rats were exposed to purified air (control), to two important constituents of the fine particle ($< 1 \mu\text{m}$ MMAD) fraction of PM10 - ammonium sulfate [SO_4^{-2}] ($70 \mu\text{g m}^{-3}$, $0.2 \mu\text{m}$ MMAD) and ammonium nitrate [NO_3^{-1}] ($350 \mu\text{g m}^{-3}$, $0.6 \mu\text{m}$ MMAD). Rats were also exposed to an important contributor to the coarse ($> 2.5 \mu\text{m}$ MMAD) mode of PM10 - resuspended road dust (300 and $900 \mu\text{g m}^{-3}$, $4.0 \mu\text{m}$ MMAD). Exposures were 4 hr per day, 4 days per week for 8 weeks. Macrophage-dependent lung defense functions (phagocytosis and respiratory burst activity) were significantly depressed by NO_3^{-1} , SO_4^{-2} and the $900 \mu\text{g m}^{-3}$ road dust exposures, compared to purified air controls. Lung permeability, as determined from measurements of total protein and albumin concentrations in bronchoalveolar lavage fluid, was significantly greater in rats exposed to SO_4^{-2} and NO_3^{-1} , but not to road dust, when compared to air-exposed controls. Quantitative histopathologic analyses included measurement of alveolar nuclear density, alveolar chord length, alveolar septal thickness and alveolar surface area. These measures showed moderate to substantial changes and, in general, the severity of the responses was in the order of $\text{NO}_3^{-1} > \text{SO}_4^{-2} > \text{road dust}$, for the concentrations used in these exposures. A count of neutrophils and macrophages in the lung sections did not reveal significant inflammatory activity following the exposures. In summary, this study demonstrated the capability of soluble and insoluble PM10 components to produce pulmonary effects following repeated exposures. Submicron PM10 components changed morphometric characteristics of the lung, depressed macrophage functions related to defenses against respiratory infections, and increased lung permeability, which could exacerbate asthma in sensitive individuals. These findings are therefore consistent with those of epidemiological studies. The study also supports the hypothesis that the fine fraction of PM10 is more toxic than the coarse fraction. (Supported by California ARB Contract No. A933-158).

CELLULAR, PHYSIOLOGIC AND IMMUNOLOGIC EFFECTS MEASURED IN RATS AFTER PM10 INHALATION IN THE PRESENCE OF OZONE. M.T. Kleinman, D.K. Bhalla, and W.J. Mautz, Department of Community and Environmental Medicine, University of California, Irvine, Irvine, CA 92717-1825.

PM10, or airborne particles less than $10\mu\text{m}$ in mass median aerodynamic diameter (MMAD), are associated with adverse effects on human health including chronic lung diseases and mortality, but the mechanisms by which these particles cause or aggravate diseases are not specifically known. PM10 represents a complex mixture, both in terms of size and chemical composition, or an aerosol-media soluble and insoluble particles. Furthermore, the ambient aerosol composition varies markedly in different locations and at different times in the same location. Regions in which residents experience relatively high PM10 exposures are often regions with high ambient levels of other pollutants, such as ozone (O_3), as well.

To test the effects of PM10 on pulmonary defenses in relation to specific cell targets, barrier-reared Sprague-Dawley rats were exposed to purified air (control), to two important constituents of the fine particle ($< 1\mu\text{m}$ MMAD) fraction of PM10 - ammonium sulfate [SO_4^{-2}] ($70\mu\text{g m}^{-3}$, $0.2\mu\text{m}$ MMAD) and ammonium nitrate [NO_3^{-1}] ($350\mu\text{g m}^{-3}$, $0.6\mu\text{m}$ MMAD). Rats were also exposed to an important contributor to the coarse ($> 1\mu\text{m}$ MMAD) mode of PM10, resuspended road dust (300 and $900\mu\text{g m}^{-3}$, $4.0\mu\text{m}$ MMAD). Exposures were 4 hr per day, 4 days per week for 8 weeks, and rats were exposed to PM10 components alone and in combination with 0.2 ppm O_3 .

Macrophage-dependent lung defense functions (antigen binding at Fc receptors and respiratory burst activity) were significantly depressed by NO_3^{-1} , SO_4^{-2} and the $900\mu\text{g m}^{-3}$ road dust exposures, compared to purified air controls. The data from rats exposed to a mixture of all 3 components + O_3 suggest additivity. Dust and SO_4^{-2} alone decreased respiratory burst activity but, in the presence of O_3 , SO_4^{-2} and NO_3^{-1} tended to increase it. Thus particles might increase risk of respiratory infections but might also exacerbate ozone-induced lung injury. The amounts of inflammatory mediators released by macrophages from pollutant exposed rats tended to increase although none of the group means were elevated significantly vs. controls. Overall these data suggest that PM10 can reduce the macrophages ability to function in the lung's defense system.

Lung permeability, as determined from measurements of albumin concentrations in bronchoalveolar lavage fluid, was significantly greater in rats exposed to high concentrations of road dust and NO_3^{-1} , but not to SO_4^{-2} , when compared to air-exposed controls. No consistent change was observed in rats exposed to either low concentrations of the PM10 components or the mixtures containing O_3 .

Quantitative histopathologic analyses included measurement of alveolar nuclear density, alveolar chord length, alveolar septal thickness and alveolar cross sectional area. These measures showed moderate to substantial changes and, in general, the severity of the responses was in the order of $\text{SO}_4^{-2} > \text{NO}_3^{-1} > \text{road dust}$ after accounting for the concentrations of these components to which rats were exposed.

Breathing pattern and minute ventilation responses consistent with mild pulmonary

irritation were present at the high concentrations of PM10 components, but not at the low concentrations. Two component mixtures of 0.2 ppm O_3 and PM10 compounds produced breathing pattern responses indicative of an O_3 effect. The multicomponent mixtures of PM10 compounds (at higher total PM10 concentration) and O_3 induced more complex breathing pattern effects suggesting irritant interactions between PM10 and O_3 effects.

In summary, this study demonstrated the capability of soluble and insoluble PM10 components to produce pulmonary effects following repeated exposures. Submicron PM10 components changed morphometric characteristics of the lung, depressed macrophage functions related to defenses against respiratory infections, and increased lung permeability, which could exacerbate asthma in sensitive individuals. These findings are therefore consistent with those of epidemiological studies. The study also supports the hypothesis that the fine fraction of PM10 is more toxic than the coarse fraction.

This toxicological study tested the effects of PM10 components in a protocol designed to simulate essential characteristics of human exposures to ambient particulate pollutants. The study shows that critical components of respiratory system defenses can be impaired by PM10 exposure and that such effects occur at environmentally relevant concentrations. This study tested only three of the possible components of PM10 in a limited set of combinations, but demonstrated significant adverse health-related changes. Other factors, which might influence PM10 toxicity, and which may be important for the evaluation of air quality standards, for example additional components such as carbonaceous material, acidic aerosols and acidic vapors, the importance of particle size, and the effects of normal activity patterns on exposure and dose, should also be investigated.

